

**PHOSPHOCITRATE: ITS CHEMICAL SYNTHESIS, CHARACTERIZATION,
NATURAL OCCURRENCE AND ROLE IN CALCIFYING SYSTEMS**

by

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ABSTRACT

A chemical synthesis of phosphocitrate (PC) was devised to facilitate investigation into its possible natural occurrence. Concomitantly, detection systems for characterising PC were developed utilizing thin layer chromatography, electrophoresis, isotachophoresis, and enzymatic and chemical PC assays. Synthesis of PC was effected by condensing 2-cyanoethyl phosphate (CEP) and triethyl citrate, followed by alkaline hydrolysis. CEP was considered to be the reagent-of-choice because of the ease of preparing [^{32}P]-CEP from [^{32}P]-inorganic phosphate enabling the production of [^{32}P]-PC. An ion-exchange chromatographic system was developed to provide ultimate purification of PC, and it was characterised by ^1H , ^{13}C and ^{31}P NMR spectroscopy.

PC was demonstrated to inhibit potently hydroxyapatite (HAP) formation being more powerful than ATP and pyrophosphate. PC was also found to inhibit strongly calcium oxalate formation, its presence modifying crystal morphology.

The unique structure of PC led to the proposal of a structure-activity relationship for inhibitors. Secondary factors influencing inhibitor power were elucidated and two groups of inhibitors were recognised. Type I inhibitors, which included PC, appeared to be crystal growth inhibitors, whereas Type II inhibitors were proposed to act by a range of mechanisms. The demonstration of a synergistic interaction between Type I and Type II inhibitors was used to support the claim that in physiological fluids, PC could act as a potentiating agent.

Comparison of a purified fraction from tissue samples with authenticated PC showed that PC occurs in kidney and liver of the rat and rabbit whilst other studies indicated that small amounts of PC were present in rabbit bone and human urine. More detailed studies indicated that PC was present, at a level of ca. 1 nmol/mg mitochondrial protein, in mitochondria, a subcellular organelle known to concentrate calcium.

Administration of [32 P]-PC demonstrated that PC was stable in blood but rapidly hydrolysed in kidney. Further, PC was rapidly taken up by bone, consistent with its physicochemical properties. These results suggested that although PC itself might be useful in treating some disorders of calcium metabolism, for other disorders, such as urolithiasis, a more stable analogue may be desirable. The nature of such analogues was discussed.

Further experimentation indicated PC was compartmentalised within mitochondria and preliminary work demonstrated that it could be biosynthesized there under the impetus of calcium loading. Data supported the proposal that compartmentalisation protected PC from the extramitochondrial lytic activity. The role of PC as a mitochondrial agent stabilising calcium phosphate deposits as amorphous spherules rather than HAP needles was described, and the effect of this stabilisation on membrane integrity, enzyme activity and calcium mobilisation was also detailed along with specific roles for PC in particular tissues, especially bone. Avenues for future research were presented and possible roles of PC in the aetiology and therapy of some calcium metabolism disorders discussed.

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Much of the material in Chapters 2-7 has been published by the candidate and his supervisors. Published papers are enclosed in the back of the thesis. The contribution of the supervisors was in the suggestion of the topic of this thesis, and in the normal supervision of the candidate's independent researches reported.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Calcium is the major cationic constituent of most mineral deposits in biological systems. Deposition can occur as a result of the normal metabolism of organisms, for example in bones, teeth and shell material (Degens, 1976; Posner and Betts, 1975; Landis and Glimcher, 1978). In addition, pathological states can result in the formation of calcareous deposits (Fleisch et al., 1965; Catalina and Cifuentes, 1970) as in, for example, atherosclerosis, and stone formation in the urinary tract. In these mineralisations calcium may be present as a carbonate, a phosphate, or an oxalate, for example as the minerals calcite or aragonite, weddellite or whewellite, or amorphous calcium phosphate, brushite or hydroxyapatite, respectively. A combination of these salts may also occur; for example dahillite is a mixture of calcium carbonate and hydroxyapatite found in bone (Degens, 1976).

Over the last fifty years calcium has been shown to influence a wide variety of cellular functions (Table 1.1) which include: platelet aggregation, glucose transport, contraction of muscle myofibrils, discharge of neurotransmitters and hormones, bioluminescence, and enzymatic activity (for example pyruvate dehydrogenase phosphatase, phosphofructokinase, fructose 1,6-diphosphatase and adenylate kinase). It is not surprising, therefore, to find calcium referred to as a "second messenger" (Rasmussen, 1977; Rasmussen and Goodman, 1977) due to its ability to affect many events in

Table 1.1 A representative sample of cellular functions regulated by calcium.*

SECRETION OF:

neurotransmitters
hormones e.g. glucagon, adenohipophysasis hormone
proteins e.g. enzyme secretion by pancreas
agents for vasodilation and antivasodilation e.g. histamine from
mast cells
electrolytes and water e.g. in kidney epithelia

CONTRACTILE MECHANISMS:

muscle myofibrils
activities associated with microtubules and/or microfilaments e.g.
cell division, flagellar motility

MEMBRANE EVENTS:

platelet aggregation
glucose transport
fluidity of membranes
action potentials

OTHER FUNCTIONS:

bioluminescence
photoreception
fertilisation

REGULATION OF SPECIFIC ENZYME SYSTEMS:

pyruvate dehydrogenase phosphatase
pyruvate dehydrogenase kinase
fructose 1,6-diphosphatase
lipases and phospholipases
prostaglandin synthesis
cyclic nucleotide phosphodiesterase

REFERENCES

Fukuda and Kameyama (1979)
Leclercq-Meyer *et al.* (1978); Moriarty (1978)
Chandler (1978)
Van Neutan and Wellens (1979)
Greger *et al.* (1978); Suki (1979)
MacLennan and Klip (1979)
Nishida (1978)
Massini *et al.* (1978)
Hellman (1979)
Gordon *et al.* (1978)
Fukuda and Kameyama (1979)
Henry and Ninio (1978)
Clark and Duncan (1978)
Lambert and Epel (1979)
Case (1980)

*Based on data given by Case (1980)

a controlled manner.

In order that calcium can control biological functions, intracellular and extracellular calcium levels must be closely regulated and today many normal calcium deposits, once thought to be inert, are regarded as in constant flux and intimately involved in calcium homeostasis (Neuman et al., 1977; Heersche et al., 1978; Eilon and Raisz, 1978). Although ultimate control must involve the molecular mechanisms responsible for deposition and re-absorption of mineral deposits, this level of control is only the last in a sequence of decreasing complexity involving (1) control at the organ level, (2) the exchange of intra- and extracellular calcium, and (3) fundamental molecular mechanisms of calcium deposition.

1.1.1 CONTROL AT THE ORGAN LEVEL

The highest level of control of calcium homeostasis involves organ function and the hormonally mediated interaction between them. The four major organs controlling calcium metabolism are the intestine - the absorption site (Ribovich and DeLuca, 1978; Marche et al., 1978; Morrissey et al., 1978), bone - the reservoir for calcium and phosphate (Thomasset et al., 1978; Dziak, 1978; Christakos and Norman, 1978), kidney - the site for excretion and reabsorption (McPartlin et al., 1978; Farrington et al., 1978; Bonjour et al., 1978), and the thyroid-parathyroid complex. This complex secretes hormones controlling calcium levels, principally calcitonin and parathyroid hormone, in response to, among other factors, calcium concentration in the serum (Habener and Kronenberg, 1978). Calcitonin is a hypocalcemic factor inhibiting bone resorption, thus allowing its deposition

(Boris et al., 1979; Messer et al., 1973; Asch, 1978; Ono et al., 1978), whereas parathyroid hormone (PTH) acts to stimulate bone resorption and to decrease renal calcium excretion (Thomas and Ramp, 1949; Broulik et al., 1978; Habener and Kronenberg, 1978). The third major hormone of calcium homeostasis is vitamin D acting via active metabolites.

It is now known that the action of vitamin D is mediated, in part, by its metabolic transformation to 1,25-dihydroxy vitamin D and perhaps other active compounds for example 24,25-dihydroxy vitamin D (Ornoy et al., 1978; Holick and Clark, 1978; DeLuca, 1979). A primary site of metabolic control is the renal mitochondrial enzyme 25-hydroxy vitamin D-1 α -hydroxylase which is affected by such factors as serum calcium, PTH and serum phosphate levels (Norman and Ross, 1979). Thus vitamin D serves to increase intestinal absorption of calcium and facilitates PTH-stimulated bone resorption. Vitamin D, calcitonin and PTH are currently thought to be the major regulatory factors in calcium homeostasis (Arnaud, 1978), although other factors can influence calcium and phosphate metabolism, including: vitamin A (Kistler, 1978, 1979), vitamin C (Parker et al., 1979), growth hormone (Koskinen et al., 1977; Aloia et al., 1977), thyroid hormone (MacIntyre et al., 1978), sex hormones (Cruess and Hong, 1978), and perhaps vitamin K (Hauschka et al., 1978; Lian et al., 1978; Lian and Friedman, 1978).

1.1.2 EXCHANGE OF INTRA- AND EXTRACELLULAR CALCIUM

At the subcellular level mechanisms of calcium homeo-

stasis involve uptake and release of calcium by membranes [for example sarcoplasmic reticulum, mitochondria and Golgi (Herbst and Deamer, 1977; Yamazaki et al., 1979; Freedman et al., 1977, 1981)] and by calcium binding proteins such as calmodulin, vitamin D inducible calcium binding protein and parvalbumin (Means and Dedman, 1980; Laouari et al., 1980; Morrissey et al., 1978; Maximov et al., 1978; Sowadski et al., 1978). In organelles, the calcium levels may rise to such an extent that calcareous deposits form there (Greenawalt et al., 1964; Appleton and Morris, 1979; Raeymaekers et al., 1981).

At the cellular level mechanisms controlling calcium homeostasis include calcium entry across plasma membranes by, for example, passive entry, pinocytosis or ATP-energised uptake; and exit of the ion by mechanisms including ATP-driven pumps, $\text{Na}^+ - \text{Ca}^{++}$ exchange and exocytosis (Best and Ford, 1978; Brinley, 1978; Moore and Pastan, 1978; Kinne et al., 1977). Another facet of control at the cellular level is the specialised activities of differentiated cells, for example of osteoblasts and osteoclasts which control, in part, bone turnover (Vignery and Baron, 1978; Lewinson and Silberman, 1978; Heersche, 1978; Daniels, 1978; Teitelbaum and Kahn, 1980).

1.1.3 FUNDAMENTAL MOLECULAR MECHANISMS OF CALCIUM DEPOSITION

Although there are many mechanisms recognised as playing roles in controlling calcium homeostasis, perhaps the most important, and certainly the most basic, level of

control is the molecular mechanism(s) governing calcium salt deposition and dissolution. However, the exact mechanism(s) are still unknown and in order to gain information that might aid definition, three sites of calcification are usually studied: (1) the bone site, (2) the urinary tract site, and (3) the intracellular site.

1.2 THE BONE SITE

The exact mineral constitution of bone remains unknown. It is not a pure hydroxyapatite (Driesens et al., 1978; Driesens and Verbeeck, 1980; Driesens, 1980), but appears to be a carbonated hydroxyapatite (Posner, 1969; Tochon-Darguy et al., 1980; Chickerur et al., 1980) or may even be a citrated hydroxyapatite (McCann, 1979; Meyer and Selinger, 1980). Recently a form of calcium phosphate amorphous to X-ray diffraction has been recognised in bone (Wheeler and Lewis, 1977; Termine et al., 1967), apparently decreasing with age (Termine and Posner, 1967). In vitro this amorphous form (ACP) is known to be the kinetic precursor of hydroxyapatite (Boskey and Posner, 1973; Meyer and Eanes, 1978a; Termine et al., 1970) and currently it is believed that ACP is also the first-formed phase in vivo (Landis and Glimcher, 1978; Posner and Betts, 1975; Posner, 1973; Betts et al., 1975; Dougherty, 1978).

In order to explain how calcium phosphate precipitates in a controlled fashion at the bone site, three models (Neuman and Neuman, 1958; Posner, 1969; Posner et al., 1978) are usually advanced:

- MODEL A: elevation of local calcium and phosphate levels to force precipitation from a supersaturated solution,
- MODEL B: creation of nucleating sites for calcification, or removal of substances blocking these sites, and
- MODEL C: removal of inhibitors of precipitation allowing mineralisation to proceed.

1.2.1 MODEL A - ELEVATION OF LOCAL CALCIUM AND PHOSPHATE LEVELS

Fluid aspirated from cartilage has been shown to contain insufficient calcium and phosphate for precipitation to occur spontaneously (Howell and Carlson, 1968), especially if ACP, which is significantly more soluble than HAP (Meyer and Eanes, 1978a, b), is indeed the first-formed phase. Thus one model of calcification envisages elevation of calcium and/or phosphate levels as the impetus for precipitation.

One method for elevating the concentration of phosphate suggested many years ago by Robison (1923) involves the hydrolysis of organic phosphoric esters at the bone site by alkaline phosphatase activity. The consequent elevation in phosphate was proposed to initiate precipitation. Acceptance of this model has been hampered by the ubiquity of alkaline phosphatases which does not therefore explain why calcification is normally limited to the bone site. Further, no phosphoric ester specific to the bone site has ever been found. In order to answer these criticisms, research has concentrated on the elucidation of any lytic activity at the bone site which might selectively cleave a particular phosphoric ester.

Pyrophosphate has been considered as a possible compound influencing calcification (Fleisch and Russell, 1970) and recently reports have described pyrophosphatase activity at the bone site (Korhonen et al., 1977; Arsenis and Huang, 1977), but it is still unclear whether this is due to a unique enzyme or is a facet of general phosphohydrolase activity (Posner, 1969).

There is, however, another method by which calcium and phosphate levels can be locally elevated: concentration of the ions by vesicular bodies. Granules have been found in soft tissue mitochondria and have been identified by micro-incineration and X-ray diffraction as ACP (Greenawalt et al., 1964). Furthermore, ACP granules have been observed in mitochondria prior to mineralisation in cartilage (Brighton and Hunt, 1978a, b; Lewinson and Silberman, 1978; Matthews et al., 1970). Chondrocyte mitochondria are known to be capable of loading up to 300 times the calcium accepted by soft tissue mitochondria and are relatively insensitive to calcium-uncoupling (Shapiro et al., 1976; Shapiro and Lee, 1978; Lee and Shapiro, 1978), highlighting the capacity of mitochondria at the bone site to concentrate calcium. Lehninger (1970) has proposed that these "micropackets" of calcium phosphate are then passed out of the cell, by a process analogous to exocytosis, for calcification. There is no direct evidence for this suggestion, but two recent avenues of research add weight to the proposal. EM observation of the intestinal translocation of calcium indicates that mitochondria accumulate at the brush border, then take up calcium and move towards the basal lamellae (Weringer et al., 1978). If an analogous process occurs in

mineralising bone, then a controlled introduction of calcium phosphate to the calcification site, as envisaged by Lehninger, is possible.

The second avenue of research implicating mitochondria in calcification has been obtained through EM observation of calcifying epiphyseal cartilage. The ACP deposits that accumulate in mitochondria disappear and simultaneously calcium phosphate deposits appear in small, vesicular bodies (Anderson and Hsu, 1978; Hsu *et al.*, 1978; Wuthier *et al.*, 1977; Schraer and Gay, 1977; Boskey, 1979; Felix *et al.*, 1978; Ali *et al.*, 1978; Dereszewski and Howell, 1978) unique to the mineralising front - the matrix vesicles. The exact source of the bodies is unknown, but since they can accumulate calcium and phosphate and can initiate calcification *in vitro* then, along with mitochondria, they are sites that can concentrate calcium in bone and may be intimately involved in controlling mineralisation.

The local elevation of concentration in vesicular bodies leading to the precipitation of calcium phosphate appears well established, but whether or not elevation of the calcium and phosphate levels in the extracellular fluid is responsible for initiating bone calcification remains unknown. Although accumulation and movement of the deposits from mitochondria to matrix vesicles is correlated with mineralisation in calcifying cartilage, its role as a causative factor has yet to be unambiguously demonstrated.

1.2.2 MODEL B - NUCLEATION OF CALCIFICATION

In another type of model proposed to explain calcific-

ation, the properties of some molecules to promote precipitation is seen as the cause of bone formation. In these models, certain molecules nucleate bone mineral by mechanisms including calcium binding or the ability to promote epitaxial growth. In the latter regard, collagen has been often discussed since it has been shown to bind ions and induce the formation of hydroxyapatite needles in the hole zone of collagen fibrils (Berthet-Colominas et al., 1979; Althoff et al., 1978; Waddell, 1972). In vitro, collagen from a wide variety of sources has been shown to have the ability to nucleate hydroxyapatite (Rey et al., 1977; Huckins, 1978; Quittner and Wadkins, 1978; Luben and Wadkins, 1971; Wadkins and Luben, 1978; Wiedner and Wilhelm, 1977). EM examination of mineralising bone indicates that hydroxyapatite does form on collagen fibrils in a regular fashion (Yamada, 1976; Lees, 1979). These data strongly suggest that collagen has a role in calcification. However, collagen is widespread in the body and it is difficult for this model to explain the specificity of calcification. Recent evidence (Hohling et al., 1980), however, has been used to argue that collagen from bone does possess some unique features associated with negatively charged amino acids acting as initiators of calcification.

Phospholipids, either as proteolipids or as complexes with calcium and phosphate, have also been suggested as nucleators influencing calcification. A proteolipid that will promote precipitation in vitro (Ennever et al., 1978) has been isolated from bacteria which calcify (Boyan-Salyers et al., 1978). Recent work has suggested that an analogous

proteolipid is found in bone (Ennever et al., 1977; Wuthier and Gore, 1977). The complexes between acidic phospholipids, calcium and phosphate are well known for their ability to promote hydroxyapatite formation in vitro (Boskey and Posner, 1977; Boskey, 1978). Acidic phospholipids that bind calcium have been isolated from bone (Vogel et al., 1978; Wuthier and Gore, 1977). It is important to note that whereas collagen is known to be elaborated before calcification (Lees, 1979; Yamada, 1976), a similar sequence with phospholipids is not yet defined.

Another class of compounds proposed to nucleate hydroxyapatite are phosphoproteins (Lee et al., 1977; Keeley, 1977). In fact, serine phosphate has been recognised in the protein present at certain calcified sites, for example, the human aorta (Keeley, 1978). Recently, however, evidence has been presented (Termine and Conn, 1976; Termine et al., 1980) indicating that, in vitro at least, phosphoproteins instead of nucleating hydroxyapatite inhibit its formation.

This anomalous type of behaviour has also been observed with another class of proteins suggested to be calcification nucleators; namely the γ -carboxyglutamate (γ -Gla) containing proteins (Hauschka et al., 1975; King, 1978; Price et al., 1976; Lian et al., 1978; Lian and Friedman, 1978). A role in controlling calcification was suggested from the observation that osteocalcin, a γ -Gla containing protein found in bone, was synthesised in chicken embryonic bone at the time of calcification (Hauschka and Reid, 1978a, b). In fact, γ -Gla containing proteins have been found in a wide variety of calcifications including

urinary stones, but a role as nucleator has been difficult to reconcile with the observation that in vitro it potentially inhibits hydroxyapatite formation (Price et al., 1976). The question also arises as to whether its appearance in calcifications is merely a reflection of its calcium-binding ability.

A regulatory role in calcium handling can be envisaged for phosphoproteins and γ -Gla containing proteins because of their inhibitory nature. Only collagen and phospholipids (either as conjugates or complexes) show clear potential as hydroxyapatite nucleators and of these two, only collagen has been shown to be synthesised and laid down before calcification.

Fleisch and Russell (1970) have suggested a modification to this basic model because of the difficulty in explaining how collagen, which is ubiquitous, could nucleate calcification at specific sites and not at others. In this model, pyrophosphate binds to nucleating sites on collagen and is specifically removed at the bone site, thus removing the barrier to hydroxyapatite formation. However, the applicability of this model is limited by the fact that pyrophosphate itself is ubiquitous and, as yet, no specific enzyme has been unambiguously demonstrated at the bone site for the selective removal of pyrophosphate (Posner, 1969), although it is labile to the general phosphohydrolytic there and at other sites (Fleisch and Russell, 1970).

1.2.3 MODEL C - REMOVAL OF INHIBITORS OF CALCIFICATION

Since the recognition that ACP may be the biological precursor of hydroxyapatite, many compounds have been shown

to inhibit hydroxyapatite formation by stabilising ACP and inhibiting the transformation of ACP into hydroxyapatite. Inhibitors include: ATP (Blumenthal et al., 1975, 1977), citrate, fructose 1,6-bisphosphate, magnesium, phosphitin (Termine and Conn, 1976; Termine et al., 1970), proteoglycans (Blumenthal et al., 1979), and poly-L-glutamate (Hay et al., 1979). In models of calcification involving inhibitors, their selective removal at the bone site is proposed to initiate calcification. Inhibitors often used in such models are organic phosphoric esters so the factors suggested to remove them are the phosphohydrolytic activities present either extracellularly in forming bone, or in the matrix vesicles.

Fleisch and Bisaz (1962) isolated a phosphate compound from urine which inhibited hydroxyapatite formation and recognised it as pyrophosphate. Since then work has established it as not only a potent inhibitor of hydroxyapatite formation but as a compound occurring at the bone site (Perkins and Walker, 1958). Pyrophosphate, however, is highly labile in vivo (Jung et al., 1970) making it difficult to envisage how it could act specifically at the bone site. Nevertheless, because for example, nothing is definitely known on the possible occurrence of high concentrations of the inhibitors in a particular microenvironment, pyrophosphate has been often suggested as a controlling influence on calcification in vivo (Hausmann et al., 1970; Fleisch and Russell, 1970, 1972; Russell et al., 1970; Fleisch et al., 1970; Neuman et al., 1978). The recent recognition of matrix vesicles at the mineralising front leads to the

possibility that these bodies may contain an enzyme specially suited for the removal of pyrophosphate. As yet, however, despite the findings that these bodies are rich in phosphatases (Fortuna et al., 1978; Felix and Fleisch, 1976), no specific pyrophosphatase activity has been revealed.

In an extension of this research motivated to take advantage of the physicochemical properties of pyrophosphate for therapy, its analogues - the diphosphonates, have been prepared. These compounds are stable to enzymatic hydrolysis (Strates et al., 1971) and have the ability to inhibit calcium salt precipitation (Fleisch et al., 1970; Russell et al., 1970) and dissolution (Robertson and Fleisch, 1970; Francis et al., 1969; Francis, 1969) by binding to the surface of the deposition (Meyer and Nancollas, 1973; Jung et al., 1973). Their use, however, in the treatment of calcium disorders such as calcium lithiasis, Paget's disease and osteolytic sarcoma, has been limited by long-term effects due to diminished bone turnover (Francis et al., 1976; Miller and Jee, 1977; Bone et al., 1979). Further, there is evidence that the analogues disrupt normal cellular metabolism (Komissarenko et al., 1977; Fast et al., 1977, 1978; Rowe and Hausmann, 1976; Bisaz et al., 1975; Miller et al., 1977; Caniggia and Gennari, 1977; Michael et al., 1972; Palmoski and Brandt, 1978; Ende, 1978).

Another phosphoric ester suggested to play a role analogous to pyrophosphate, is ATP (Leonard and Scullin, 1969). Again its ubiquity has made such a model of calcification difficult to accept. Because pyrophosphate and ATP release phosphate when their inhibitory effect is abolished

by hydrolysis they could possibly act to elevate local phosphate concentrations; so if this model of calcification becomes tenable these types of inhibitors could be foreseen to have a dual action. Unhydrolysed, they inhibit hydroxyapatite formation: hydrolysed, one of their cleavage products is a precursor of hydroxyapatite.

Proteoglycans do not contain anionic phosphate groups that are often associated with inhibitory activity and in vitro show a different type of inhibition (Blumenthal et al., 1979) of the transformation of ACP into hydroxyapatite. Inhibitors usually delay the onset and sometimes the extent of the conversion process; proteoglycans do not delay the onset but they do decrease the rate of conversion and its extent. This occurs at high concentrations approximating to those found in cartilage. However, some workers consider proteoglycans to be extracellular matrices for deposition (Hohling et al., 1980; Reddi et al., 1978). Whatever the exact function of proteoglycans in the mineralising cartilage it must be shown that proteoglycans from uncalcified cartilage are different from those in calcified areas. In fact, some evidence has been presented to this effect (Lohmander and Hjerpe, 1975), but the exact role of proteoglycans in mineralisation remains obscure.

Clearly then there are many compounds which can regulate calcium phosphate formation through an inhibitory action. Models using inhibitors to explain bone formation, however, have been unable to convincingly demonstrate that their effect could be localised to the calcifying site. Research into the phosphohydrolytic activity at the bone

site and more particularly in matrix vesicles may lead to an understanding of the role that inhibitors play in bone formation.

1.3 URINARY TRACT SITE

Calculi found in the urinary tract are composed of various salts including calcium oxalate, calcium phosphate, uric acid, magnesium ammonium phosphate and/or cystine (King, 1971). Most stones are predominantly calcium oxalate but a mixed composition is frequently found (Kennoki *et al.*, 1978; Robertson *et al.*, 1978; Pellegrino and Biltz, 1978; Gonzalez-Diaz *et al.*, 1979). In addition to the crystalline matter, a matrix of protein or mucoprotein is always found (King, 1971; Resnick and Boyce, 1978; Rao *et al.*, 1978).

With regard to calcium lithiasis, Pak (1978) has noted that many broad aspects of their formation are analogous to the mechanisms suggested to explain bone deposition. Consequently, factors influencing lithiasis can be considered in the three main groups proposed for bone formation:

- MODEL A: calcium and phosphate levels - dependent on cellular and hormonal status,
- MODEL B: nucleation (promotion) - inorganic or organic substrates acting to promote the deposition of stone salts, and
- MODEL C: inhibitors - compounds which inhibit nucleation, growth and/or aggregation of calcium salts.

1.3.1 MODEL A - ELEVATION OF LOCAL CALCIUM AND PHOSPHATE LEVELS

Studies on the urine of stone formers indicates that

it is nearly always supersaturated with regard to stone components (Fleisch, 1978; Robertson et al., 1971). However, non-stone formers quite often excrete urine that is also supersaturated (Gill et al., 1974; Finlayson, 1978). Therefore, there tends to be disagreement as to whether the urine of stone formers is more supersaturated than that of normal individuals. Pak and Holt (1976), for example, found that the activity product ratios of brushite and calcium oxalate were elevated in stone-forming groups due mainly to a high renal excretion of calcium. However, Crassweller et al., (1978) found that whilst calcium phosphate supersaturation was greater in stone-formers, levels of saturation of urine with calcium oxalate were identical in stone-formers and normal individuals. Consequently, Finlayson (1978) has suggested that stone formation may arise from a difference in boundary conditions (that is conditions resulting in metastability of urine), or the stochastic nature of the process, urinary inhibition or some other process. Robertson et al. (1978) have attempted to isolate the factors predisposing an individual to form stones and have found that apart from factors influencing saturation of urine, other elements including inhibitors and promoters also influence lithiasis. Factors requiring consideration include: (1) calcium, (2) oxalate, (3) pH, (4) acid mucopolysaccharide, and (5) uric acid. More recently Hautmann et al. (1980) investigated the concentrations of calcium and oxalate in the cortex, medulla and renal papillae. Whereas the ion product was $6 \times 10^{-7} \text{ M}^2$ in the cortex, medulla and urine (approximately the formation product of calcium oxalate), in renal papillae it was $1 \times$

10^{-4} M^2 . Strictly activities should have been used to allow for the effect of chelation. Nevertheless, the high relative concentration of calcium and oxalate in renal papillae ($\times 1000$) does raise the question as to why lithiasis does not proceed in normal individuals and highlights the role that inhibitors must play to prevent stone formation in the urinary tract.

1.3.2 MODEL B - NUCLEATION OF STONES

The organic matrix of stones is well known (Rao et al., 1978; Delatte et al., 1976; Boyce and King, 1959) and its role as an etiologic factor has been discussed. However, despite the fact proteins, which can promote crystallisation (Boyce et al., 1954), are raised in concentration in the urine of stone-formers, the role of promoters has been largely ignored until recently (Delatte et al., 1976), presumably because inclusion of organic matrices into stones could be a secondary effect. Calcium binding or irritation of the urinary tract could account for their presence.

After the recognition of a new amino acid, γ -carboxy-glutamate (Hauschka et al., 1975, 1978), came the discovery of γ -Gla containing proteins in many calcifications including bone and calcium stones (Hauschka and Reid, 1978a). These proteins strongly bind calcium and were proposed to act, therefore, as a centre for subsequent crystal growth (Lian and Friedman, 1978). In vitro, however, γ -Gla containing proteins strongly inhibit the formation of hydroxyapatite (Hauschka and Reid, 1978b) so the role of these calcium binding proteins in the promotion of calcium lithiasis is undefined.

Recently data has been published which re-establishes the importance of a nucleating agent in the etiology of stone formation. Hallson and Rose (1979) concluded from studies on the effect of ultrafiltered urine on precipitation that human urine contains macromolecules which promote both the formation of calcium oxalate and calcium phosphate crystals. Notably the ultrafiltrate generally failed to develop crystals, whereas addition of the retained uromucoid promoted precipitation.

The role of organic components in nucleating precipitation of calcium salts remains controversial but the phenomenon of epitaxis, in which crystals of one salt induces the growth of another type of salt, has become established and is currently the focus of much attention. Lonsdale (1968) has tabulated the dimensions of the common stone-forming compounds in their usual habits and has shown that the geometrical fit between the respective lattice-works is very close in a large number of cases. This was suggested to account for the common observation that stones are of mixed composition. Since then, in vitro studies have established that brushite will nucleate calcium oxalate (Pak and Holt, 1976), and that monosodium urate monohydrate nucleates calcium phosphate and calcium oxalate (Pak et al., 1976; Coe et al., 1975; Pak and Arnold, 1975). This latter finding emphasizes the role that excessive urate excretion, one of the risk factors given by Robertson et al., (1978), could play in calcium lithiasis. Further evidence for the role of urate comes from the observation that lowering the hyperuricosuria that often accompanies calcium urolithiasis reduces the

formation of calcium stones (Coe, 1978).

Nucleation of calcium salt precipitation by organic components, although possible in vitro, remains as with bone formation, of unclear significance in vivo. Epitaxial induction by stone components, however, is not only well established in vitro but explains several observations including the mixed composition of stones and the role of urate excretion in calcium lithiasis.

1.3.3 MODEL C - INHIBITORS OF STONE FORMATION

The supersaturation generally found in urine and the presence of nucleators leads to the question as to why stones do not form more often. Consequently one line of research has centred on compounds present in urine that act to inhibit the deposition of stone salts.

Inhibitors can act to retard crystal growth after nucleation and/or to inhibit the aggregation of crystals. In addition, when calcium phosphate deposition is involved, compounds can act to stabilise ACP formation, thus inhibiting the crystallisation of hydroxyapatite.

Inhibitors that have been found in urine and tested in vitro include citrate, pyrophosphate (Meyer and Smith, 1975a, b), magnesium (Fleisch and Bisaz, 1964), acidic polypeptides (Nakagawa et al., 1978), ribonucleotides (Ito and Coe, 1977), and glycosaminoglycans (Sallis and Lumley, 1979). Trace metals (Cu^{++} , Zn^{II} , Sn^{II} and Al^{III}) found in urine are not considered to be of major importance as inhibitors (Meyer and Angino, 1977). Although it has been claimed (Sutor et al., 1978, 1979a, b; Sutor and Percival, 1978) that an artificial urine possesses an equal inhibitory action

to urine samples, this is not a generally accepted view. For example, Bisaz et al. (1978) have attributed 77% of the inhibitory potential of urine to citrate, pyrophosphate and magnesium, concluding that inhibitors of an as yet unknown nature may be present. Rose (1975) noted that an artificial urine composed of 12 solutes did not have the full inhibitory effect of normal urine. However, it should be noted that the quantitation of inhibitory activity in urine is a controversial subject. It is a relatively simple task to test a compound for inhibitory activity but it has proved difficult to devise systems that allow quantitation, and enable the activity product for calcium and phosphate in urine samples to be measured and related to that sample's tendency for stone salt formation (Fleisch, 1978; Finlayson, 1978).

There is some evidence that normal individuals excrete a urine that contains more inhibitory units than stone-formers urine (Singla et al., 1978; Howard, 1976), but there is no consensus as to which inhibitory agent may be depleted in stone-formers urine. Pyrophosphate and peptides have been tested (Lewis et al., 1966; Russell and Hodgkinson, 1966; King and Boyce, 1962; Boyce and King, 1963; Boyce et al., 1962) but no unambiguous results have emerged. Since stone-formers have been found to excrete larger crystals than normal individuals in response to an augmented oxalate supply (Robertson and Peacock, 1972; Robertson et al., 1971), some research has focussed on the urinary glycosaminoglycans, potent crystal growth inhibitors, as a risk factor in the etiology of stone disease (Robertson et al., 1978).

Urine contains substances inhibiting stone salt precipitation and subsequent crystal growth and aggregation, but the exact contribution of each inhibitor to calcium phosphate and calcium oxalate formation remains uncertain as does the role of inhibitors in stone disease. Indeed, not all the inhibitors in urine may have been recognised yet.

1.4 INTRACELLULAR SITE

Calcium is distributed unequally within the cell but attempts to elucidate its occurrence in the cellular compartments have been hampered by the damage caused on disruption of the cell. Nevertheless, evidence has accumulated indicating that mitochondria act as a "sink" for intracellular calcium (Bygrave, 1978; Fiskum and Lehninger, 1980). Mitochondria from various sources are now known to take up calcium in a process dependent on respiration and linked to H^+ -efflux (Yamazaki et al., 1979; Revis and Marusic, 1979; Panfili et al., 1976; Blondin et al., 1977; Tew, 1977; Harris, 1978). Further, in response to external conditions calcium will be released from mitochondria (Shapiro and Lee, 1978; Hamilton and Holdsworth, 1970; Luthra and Olson, 1976). During the loading of calcium into mitochondria the appearance of granules in the matrix has been noted and these are now known to be deposits of ACP (Greenawalt et al., 1964; Ostrowski et al., 1975; Becker et al., 1974). Notably, hydroxyapatite, the more insoluble phase, is not deposited and consequently the soluble calcium levels in the mitochondria must be higher than if hydroxyapatite had formed. This must affect the metabolic state of the mitochondria, since calcium levels modulate this (Denton et al., 1980).

Apart from this effect, the organelle can more readily mobilise calcium if ACP is precipitated since the amorphous phase is more labile than hydroxyapatite (Termine and Eanes, 1972).

The foregoing observations indicate that in the deposition of mitochondrial calcium phosphate, calcium loading is a crucial factor. Unlike bone and stone formation, the elevation of calcium levels can be accepted as a causative factor. However, in common with these other two calcifying systems, the role of inhibitors is a promising area. Since only ACP forms in mitochondria the question arises as to the nature of the factors responsible for this. In this regard, ATP and magnesium have recently been suggested to play a role (Blumenthal et al., 1977), but in general the phenomenon of intramitochondrial calcification is not well understood. For example, the question of nucleation in mitochondrial calcification has not been examined.

1.5 CONCLUSIONS AND OUTLINE OF RESEARCH PROGRAMME

The above discussion on the possible mechanisms of calcification at the various sites indicates that of the three general models A, B and C (elevation of calcium and phosphate levels, nucleation, and inhibition), the most acceptable model and the one most clearly demonstrated to influence all calcifying systems is inhibition. Calcium and phosphate levels, whilst implicated in mitochondrial calcification, are of unknown significance in bone mineralisation and in the etiology of urinary and renal stones. Similarly, the role of nucleation is unclear. However, although the exact role and complete chemical identity of inhibitors is not known,

observations clearly indicate that they influence calcification to some degree. At the bone site, for example, ACP has been detected so inhibitors must act to stabilise this form. Similarly, ACP has been demonstrated to be deposited in mitochondria and must be stabilised by inhibitors. In urine, which is usually supersaturated with respect to stone salts, the role that inhibitors must play is again emphasised. Many inhibitors have already been identified at calcifying sites such as ATP, citrate, pyrophosphate, glycosaminoglycans and magnesium, but none of these provide a fully acceptable model of calcification. In order to explain, in detail, calcification and especially the role that inhibitors play in this process, much work has been done to recognise new inhibitors of calcification.

The identification of any new inhibitor that might enable a more detailed explanation of calcification to be deduced has necessitated research in this area usually to involve purification of samples from calcifying sites, recognition of inhibitory activity in particular fractions, and identification of the inhibitory agent(s) in that fraction. In this way it has been shown that, for example, a group of salivary proteins, including statherin, potentially inhibit calcium phosphate (Hay et al., 1979; Schlesinger and Hay, 1977; Wong and Bennick, 1980). Similarly, protein inhibitors from urine have also been found (Nakagawa et al., 1978), and there is a continuous search for new and more potent inhibitors.

Perhaps the most promising of recent developments started from the work of Howard et al. (1967) who isolated

a potent inhibitor, in an impure form, from urine and suggested it was peptidic. Later work (Barker et al., 1970) found that it was stable in 6N HCl at 110°C for at least 72hr suggesting the compound was not of peptidic nature. Smith et al. (1973) substantiated this claim and found that the inhibitor had a molecular weight of less than 1000 and was anionic and stable to enzymatic digestion including trypsin, intestinal alkaline phosphatase and crude papain.

In a similar type of study Thomas (1971) purified urine from stone formers and healthy individuals by ion exchange chromatography. Two inhibitory peaks were detected, the first containing organic phosphates and pyrophosphate. The second peak contained citrate and other inhibitors resistant to hydrolysis. It was suggested that the second peak was decreased in patients with idiopathic renal lithiasis but the nature of the compounds in this peak remained unknown.

In continued investigation into the nature of the compound(s), Howard (1976) reported that acidification of a solution of phosphate, citrate and calcium followed by neutralisation led to a great increase in inhibitory potential of the mixture. "Factor X" formed by this in vitro reaction was postulated to be present in biological samples but the identity of "factor X" and indeed its very existence in nature was obscured by the fact that the ion-exchange procedure used to isolate it from urine also caused its artefactual formation. Howard (1976) tentatively postulated that "factor X" which contained phosphate and citrate, might well be a new compound "phosphocitrate". Using an impure, chemically

synthesised preparation of phosphocitrate, inhibitory ability towards calcium phosphate formation was demonstrated. The impurity of the sample, however, meant that the chemical nature of "factor X" could not be unambiguously assigned. Further, complications arose when earlier work by Meyer et al. (1959) on the synthesis and lability of phosphocitrate was considered. In this isolated publication, which did not lead to any subsequent reports, phosphocitrate was demonstrated to be completely hydrolysed within 4hr in 0.1N HCl at 100 °C. Clearly this lability data did not agree with the lability of the inhibitor previously described (Howard et al., 1967; Howard, 1976; Barker et al., 1970). Thus, although research suggested a new inhibitor was present in urine, its exact identity and indeed its very existence was still speculative, partly due to artefactual formation during its purification.

The role of "phosphocitrate" was unclear mainly because no authenticated phosphocitrate had been tested for anticalcifying activity, nor had any been used for comparison with "factor X". Indeed, Sutor et al. (1979a) had even questioned Howard's assertion that acidification and neutralisation of a solution of phosphate and citrate could produce phosphocitrate. Lehninger (1977) reported in a review on the possible role of mitochondria in calcification that, although phosphocitrate was a potentially important new inhibitor, unambiguous identification awaited use of a high-yield organic synthesis for the purpose. It was suggested, however, that "factor X" was also present in mitochondria acting to stabilise the "micropackets" of calcium phosphate

that formed as the amorphous phase. As a cautionary note it was added that the inhibition shown by the biological fraction could be due to an impurity migrating with the postulated phosphocitrate.

In view of the preceeding comments, work described in this thesis represents an attempt to seek confirmation of earlier proposals on the identity of the inhibitor and to extend work into aspects of its physiological role(s) and general biochemistry. Initially an attempt was made to develop an alternative synthesis of phosphocitrate and to characterise it for use as a standard and general biological probe. As a consequence, the availability of this reference compound was foreseen as enabling the development of analytical systems for the investigation into the possible natural occurrence of phosphocitrate. For phosphocitrate to be of value as an inhibitor of calcification it should affect the crystallisation of both calcium phosphate and calcium oxalate. This thesis reports on such studies and will consider the broader question of why certain compounds are more potent inhibitors than others.

Finally, the possibility of the natural occurrence of phosphocitrate raises questions as to its lability in vivo at calcifying sites, its biosynthesis and subcellular distribution. These aspects will be discussed with reference to physiological role(s) that these data may support. In addition, the potential of synthetic phosphocitrate for use as a therapeutic agent in controlling some disorders of calcium metabolism will be considered with reference to information on its lability in vivo.

CHAPTER 2

SYNTHESIS AND CHARACTERISATION OF PHOSPHOCITRATE

2.1 INTRODUCTION

Any investigation into the possible natural occurrence of phosphocitrate (PC) or of its physicochemical properties first requires that a pure compound be available as a reference material. As a further consideration, isotopically labelled PC would be of potential value in elucidating the metabolism of PC in biological systems.

For these criteria to be met, two approaches to the synthesis of PC were therefore indicated: either a high-yield synthesis enabling [^{14}C]-citrate to be used for labelling, or a method allowing the use of [^{32}P]-phosphoric acid which, in comparison to other radiochemical materials, is relatively inexpensive to purchase and can be obtained in high specific activity.

The only previous synthesis of PC reported was that described by Meyer et al. (1959) which unfortunately cannot satisfy the above criteria. The method was of low yield and the phosphorylating reagent was dibenzyl chlorophosphate which is difficult to label with phosphorus-32. Further, the synthesis, as described, required special equipment: a molecular still. The low yield may have been due partly to the tertiary sterically-hindered hydroxyl group of the starting chemical, triethyl citrate, which would effectively block reaction with the phosphorylating reagent. Since the attempt by Meyer et al. (1959), an increased range of more powerful phosphorylating reagents has become available

(Slotin, 1977; Zhdanov and Zhenadarova, 1975) and of these two o-phenylene chlorophosphate and cyanoethyl phosphate appeared to meet the criteria expressed.

o-Phenylene chlorophosphate (o-PCP) is considered now to be one of the most potent phosphorylating reagents available; it reacts rapidly even with tertiary or sterically-hindered hydroxyl groups (Khwaja et al., 1970; Khwaja and Reese, 1971), and was therefore selected for evaluation. Although it is not easy to label o-PCP with phosphorus-32, since the reagent must be synthesized from relatively expensive [^{32}P]-PCl by a lengthy method, nevertheless the possibility of a high-yield might enable [^{14}C]-labelling. The basis of the synthetic pathway (Figure 2.1) involves three steps: (1) coupling of o-PCP with an alcohol (ROH); (2) cleavage of the heterocyclic ring by the addition of water; and (3) removal of the protecting group by either reductive cleavage or oxidation.

Another phosphorylating reagent, 2-cyanoethyl phosphate (CEP), has been used to prepare [^{32}P]-labelled nucleotides (Tener, 1961) and since the method employs [^{32}P]-phosphoric acid, it is now frequently used for this purpose (Slotin, 1977; Hecht et al., 1973). Figure 2.2 outlines the steps involved in this synthesis which requires prior activation of the pyridinium salt of CEP with dicyclohexylcarbodi-imide (DCC) giving an adduct that will react with an alcohol. Alkaline hydrolysis of the protecting group gives the phosphoric ester, and the aqueous conditions also decompose unreacted DCC. The simple preparation of [^{32}P]-CEP from [^{32}P]- P_i indicated it was another reagent worth evaluating.

Figure 2.1 Synthesis of phosphoric esters using o-phenylene chlorophosphate

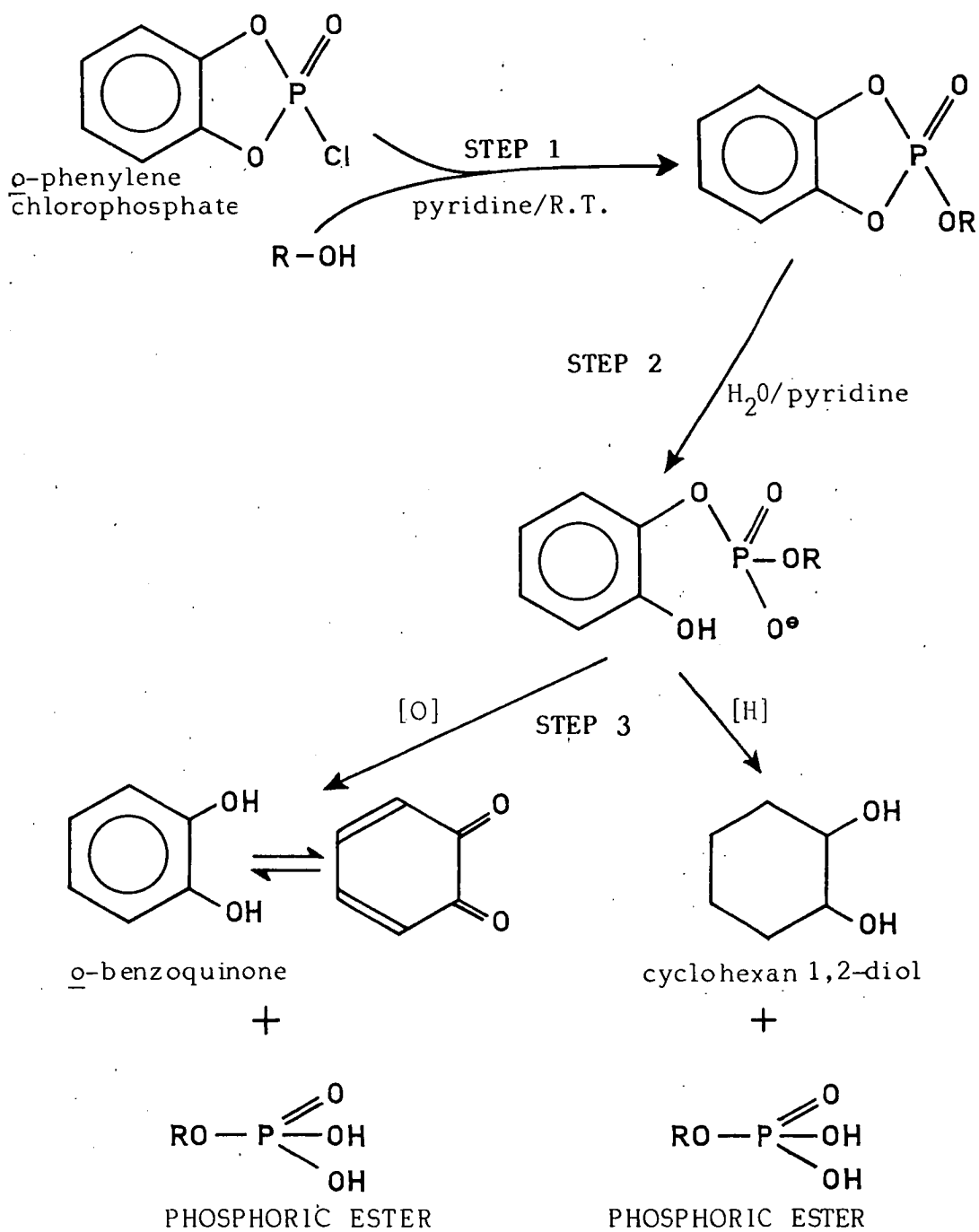
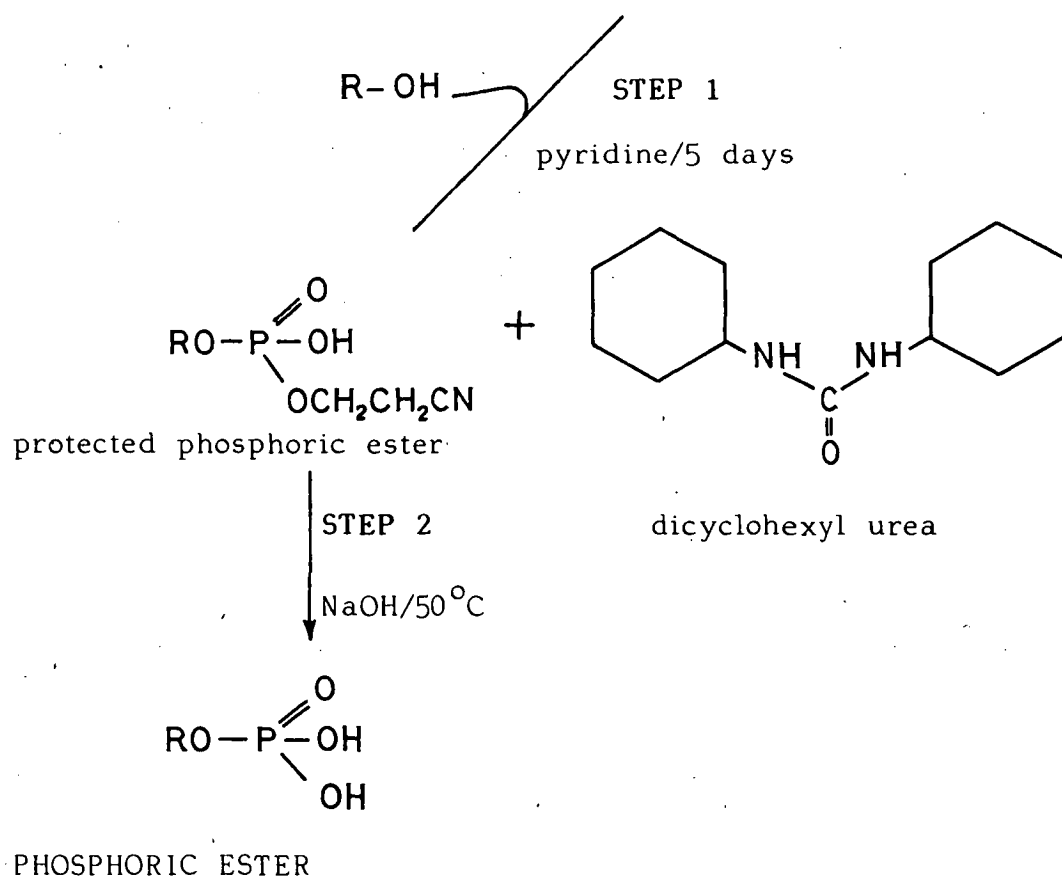
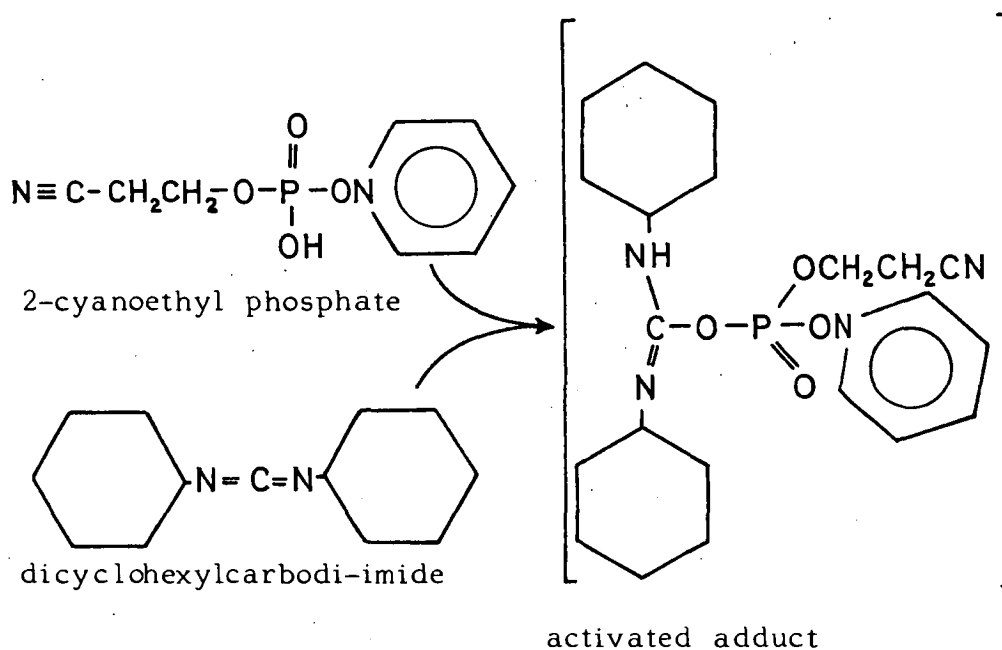


Figure 2.2 Synthesis of phosphoric esters using 2-cyanoethyl phosphate



Although synthesis of PC might be achieved by either of these routes, separation of products and ultimate purification of PC presents additional problems. The initial synthesis of PC described by Meyer et al. (1959) used an ion-exchange chromatographic system for purification of the product. The use of formate as the buffer anion obviously led to poor separation and further, the calcium salt of PC had to be prepared after chromatography in order to separate it from the buffer eluant. One of the objects of preparing PC was to test it for its anti-calcifying ability - as evaluated by the prevention of calcium phosphate or calcium oxalate precipitation - so it was desirable to be left with a salt uncontaminated with divalent metal ions, which could possibly interfere with the calcification systems.

As another consideration, investigation by Howard (1976) has suggested that acidic conditions may lead to artefactual production of PC in a low yield. Although this is not a consideration in the isolation of chemically-synthesized PC it does imply, that for any other subsequent attempt to quantitatively isolate PC from biological sources, an ion-exchange system operating at neutral pH is desirable to eliminate, if possible, artefact formation.

The high binding capacity of ion-exchange resins compared with cellulose or silica based systems suggested it was reasonable to use the former type of chromatography, but desirable to try a more strongly binding anion for eluting citrate and PC with the aim of effecting a better separation of these anions. Consequently, various ion-exchange systems were investigated during attempts to prepare PC.

2.2 MATERIALS

Triethylcitrate and hydracrylonitrile were purchased from Fluka Chemical Co., Buchs, Switzerland and o-phenylene chlorophosphate was a product of Aldrich Chemical Co., Milwaukee, Wisconsin. All other chemicals were reagent grade and obtained from usual commercial sources.

Before use, tetrahydrofuran was dehydrated by passage through a basic alumina column (Woelm, Eschwege, Germany) and stored over molecular sieve 4A. Pyridine was refluxed over KOH pellets and recovered by distillation. The resins AG 1-X8 (100-200 mesh), chloride form, and AG 50-W-X8 (200-400 mesh), hydrogen form, were products of Bio-Rad Laboratories, Richmond, California. The resins were cycled before use with appropriate solutions (Bio-Rad Laboratories, 1976) and the AG 1-X8 resin converted to either the nitrate or bicarbonate form.

Wheat germ acid phosphatase (E.C. 3.1.3.2; 0.4U/mg protein) was from the Sigma Chem. Co., Sydney, Australia, and alkaline phosphatase (E.C. 3.1.3.1; 400U/0.17ml; calf intestine, Grade I) was a product of Boehringer-Mannheim, Melbourne, Australia.

2.3 METHODS

2.3.1 CHEMICAL ANALYSES

2.3.1.1 Citrate

Citrate was determined by degradation to pentabromoacetone as described by Taylor (1953). Accordingly 5ml of 27N H_2SO_4 was added to 5ml of the test solution and after

mixing, 5ml of an oxidising-brominating mixture containing 19.84g KBr, 5.44g KBrO_3 and 12.00g NH_4VO_3 in 1 litre was added.

The tubes were allowed to stand for 30 minutes; then 2ml of freshly prepared ferrous sulphate solution (22.0% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 1N H_2SO_4) was added to decompose unreacted bromine. The tubes were mixed and left for 10 minutes. Six ml of light petroleum spirit (60-80°C b.p.) was added and each tube was mixed vigorously for 1 min before the green, aqueous layer was carefully removed by vacuum aspiration. Sufficient anhydrous sodium sulphate was then added to completely absorb any aqueous phase remaining, and the organic phase was poured into clean, chilled test tubes. A 5ml portion was pipetted into a test tube containing 3ml of freshly prepared 2% Na_2S and each tube shaken for 1 min. The top, organic layer was removed and the yellow, aqueous phase colorimetrically determined at 450nm within 10-20 min. Standards of 0-200 μg citrate were included routinely.

2.3.1.2 Phosphate

Inorganic phosphate (P_i) was estimated by the Fiske-SubbaRow method (Fiske and SubbaRow 1925, 1929). One ml of 5N H_2SO_4 and 1ml of 2.5% ammonium molybdate was added to 8ml of sample. After mixing 100 μl of a reducing solution (2.5% of mixture comprising 1g 1-amino-2-naphthol sulphonic acid, 6g $\text{Na}_2\text{S}_2\text{O}_3$, 6g Na_2SO_5) was added, the tubes mixed and allowed to stand for 15-30 min. Colour development was measured at 620nm and standards of 0-2 μmcl KH_2PO_4 were included routinely.

2.3.1.3 Phosphocitrate

Based on the published data of Meyer et al. (1959) regarding the lability of PC and subsequently confirmed here, PC was analysed for citrate or P_i following a 4h hydrolysis at 100°C either at a neutral pH or in the presence of $0.1\text{N H}_2\text{SO}_4$.

2.3.2 HIGH-VOLTAGE PAPER ELECTROPHORESIS

Khwaja et al. (1970) have reported a buffer for use in high-voltage electrophoresis in conjunction with their synthetic work using o-PCP. The buffer used was 0.05M tri-ethanolamine bicarbonate (pH 7.5). High-voltage electrophoresis was run on 3MM Whatman paper for $1\frac{1}{2}$ -2h before the paper was removed and air-dried prior to staining procedures.

2.3.3 STAINING TECHNIQUES

2.3.3.1 Citrate

Citrate was visualised by either the method of Higgins and von Brand (1966) or using dimethylaminobenzaldehyde (DMAB).

(a) The method described by Higgins and von Brand (1966) involves spraying the dried electrophoretogram with a solution of aniline/ribose (1.0ml aniline in 97ml of methanol was added to 1g of ribose dissolved in 3ml of water) and heating at 120°C for 5 min. Krebs cycle acids gave brown spots on a light background.

(b) A dimethylaminobenzaldehyde (DMAB) reagent (Zweig and Sherma, 1972) (1g DMAB in 10ml acetic anhydride and 90ml acetone) was sprayed onto the dried electrophoretogram. Heating at 100°C for ca. 5 min revealed citrate as a red spot on a light yellow background.

2.3.3.2 Phosphate

Phosphate and its esters were visualised by a number of different methods all involving the initial reaction between P_i and ammonium molybdate but differing in the manner of reducing the complex to give a coloured spot.

(a) Quinine sulphate (0.05g) in 100ml water was mixed with 0.5g ammonium molybdate in 400ml water followed by the addition of 4ml conc. HNO_3 . After spraying the dried electrophoretogram, P_i and phosphoric esters were observed as dark spots on a light, fluorescent background under UV light (Dawson et al., 1969).

(b) Ammonium molybdate (5g) dissolved in a mixture of 90ml acetone and 5ml each of conc. HCl and $HClO_4$ was sprayed onto the dried paper before heating at 80 °C for 3 min (Dawson et al., 1969). Phosphate was seen as a yellow spot and subsequent irradiation with UV light visualised phosphoric esters as blue spots.

(c) The electrophoretogram was sprayed with a reagent comprising 5% ammonium molybdate in 90ml of acetone, 5ml of conc. HCl and 5ml of conc. $HClO_4$ before heating at 80 °C for 3 min. A further spray with 0.5% benzidine in dilute acetic acid (90:10 H_2O /glacial acetic acid v/v) followed by drying and exposure to ammonia fumes revealed P_i and its esters as blue spots (Zweig and Sherma, 1972).

2.3.3.3 Phosphocitrate

It was not possible to visualise PC using either citrate or P_i sprays. However, as demonstrated here, PC is labile to the action of phosphatases. Thus it was possible to stain PC after electrophoresis and subsequent hydrolysis

with acid phosphatase using methods for citrate or phosphate. Accordingly, dried electrophoretograms were treated as described by Fletcher and Malpress (1953), giving an initial spray with a solution of 5% $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ in 80% EtOH. After air drying, the paper was sprayed with a buffer comprising 15mg acid phosphatase in 1.0ml of 0.1M sodium acetate buffer (pH 4.8). The paper was enclosed between two plates to retain moisture and incubated at 37°C for 1h. Phosphate was then visualised using one of the sprays detailed above.

2.4 RESULTS

2.4.1 SYNTHESIS OF PHOSPHOCITRATE

2.4.1.1 Synthesis using o-phenylene chlorophosphate

The initial steps were based on the work of Khwaja et al. (1970). A flask equipped with a pressure equalising dropping funnel and silica gel drying tube was charged with 1.37g triethyl citrate (TEC) and 0.9ml of anhydrous pyridine in 2ml of tetrahydrofuran (THF). A sample of o-PCP (0.98g) dissolved in 3ml of THF was added dropwise over 15min to the cooled, mechanically stirred solution during which time a white precipitate of pyridinium hydrochloride formed. The precipitate was separated by filtration and 0.25ml of water was added to the filtrate. After standing for 10 min, excess pyridine and water was evaporated off by rotary vacuum evaporation. Electrophoretic analysis indicated that three phosphate-containing components were present in the reaction mixture (Figure 2.3), although two of these were only minor components on the basis of the intensity of staining. A comparison with electrophoretograms of P_i and o-hydro-

Figure 2.3 High-voltage electrophoretogram of the reaction-mixture after coupling *o*-PCP with TEC.

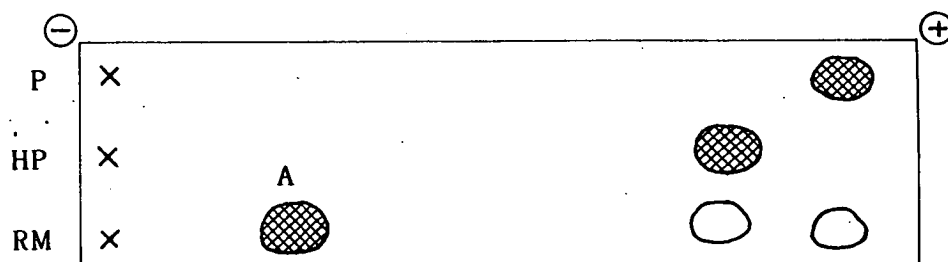


Figure 2.4 High-voltage electrophoretogram of oxidised reaction mixture

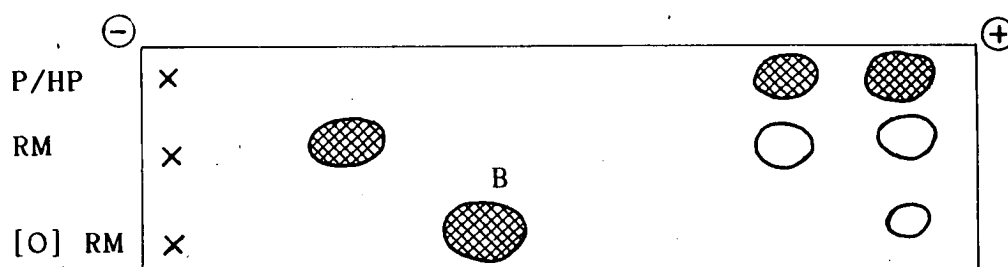
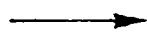
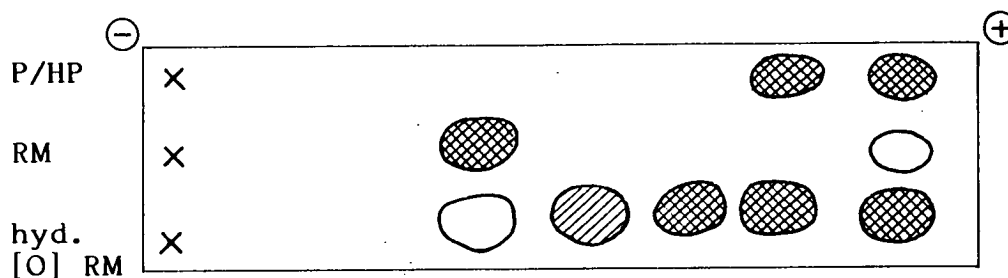


Figure 2.5 High-voltage electrophoretogram of partly hydrolysed oxidised reaction mixture.



× origin



intense spot



faint spot

- P inorganic phosphate
 HP *o*-hydroxyphenyl phosphate
 RM reaction mixture
 [O] RM reaction mixture after oxidation
 hyd. [O] RM oxidised reaction mixture after brief alkaline hydrolysis

xyphenyl phosphate (formed by the synthetic reaction after omitting TEC) indicated that these were the two major components of the reaction mixture. The presence of the slowest migrating material (Compound A), which was the major component, was taken as evidence for the successful production of the desired condensation product.

The next step involved the removal of the protecting group. Oxidation was chosen since reductive cleavage requires platinum oxide which is an expensive reagent. Oxidation, however, by any of the three methods listed by Khwaja et al. (1970) led to the formation of darkly coloured solutions with gummy material often deposited. Despite these difficulties electrophoretic analysis (Figure 2.4) indicated that only two phosphate-containing components were present: P_i and presumably phospho-triethyl citrate (Compound B). The major compound migrated in front of the protected condensation product (Compound A), perhaps reflecting an increase in charge and a decrease in molecular size. This interpretation was substantiated by analysis of the oxidised reaction mixture after subsequent exposure to alkaline conditions since P_i and four phosphoric esters were detected (Figure 2.5); the esters perhaps correspond to phosphocitrate and its mono-, di- and tri-ethyl esters.

Meyer et al. (1959) had originally described a mild method for the removal of the ester groups of triethyl citrate involving the precipitation of calcium phosphocitrate. Consequently a two-fold excess of both 0.1M NaOH and $CaCl_2 \cdot 6H_2O$ was added to phospho-triethyl citrate and the mixture left to stir overnight. The calcium salt that formed was dark

coloured and after decalcification with Dowex 50W-X8 (H^+ form) the solution that was obtained was still dark coloured. The dye appeared to impair electrophoretic analysis since definitive resolution was not achieved. If this solution was loaded onto an AG 1-X8 column in either the nitrate, bicarbonate or formic form a dark band formed. As shown in Figure 2.6, chromatography on an AG 1-X8 nitrate column using batch elution with $NaNO_3$ solutions allowed resolution of P_i and citrate. However, attempts made to separate components of a decalcified solution were unsuccessful as shown in Figure 2.7; separation of components was not obtained.

In an effort to determine the cause of this difficulty, $[^{32}P]-P_i$ was added to the coloured, decalcified solution. After column chromatography, <60% of the radioactivity was recovered. Thus the dye appeared to interfere not only with resolution but also with the recovery of compounds. Khwaja *et al.* (1970) decolourised their solutions with activated charcoal. However, use of this approach led to adsorption of the product (Figure 2.8).

Quinone material in plant extracts has been successfully removed using Florisil, Fuller's Earth or Celite (Ternynck and Avrameas, 1976; Fafunso and Byers, 1977; Sadler and Shaw, 1978; Newbury and Possingham, 1977; Hagerman and Butler, 1978). However, it has been reported that whilst Florisil efficiently removes UV impurities leaving citrate unbound, phosphorylated sugars and nucleotides are not quantitatively recovered. In any event, none of these adsorbents sufficiently removed the dye from the decalcified solution (Table 2.1).

Figure 2.6 Resolution of P_i and citrate using an AG 1-X8 (NO_3^-) ion-exchange system

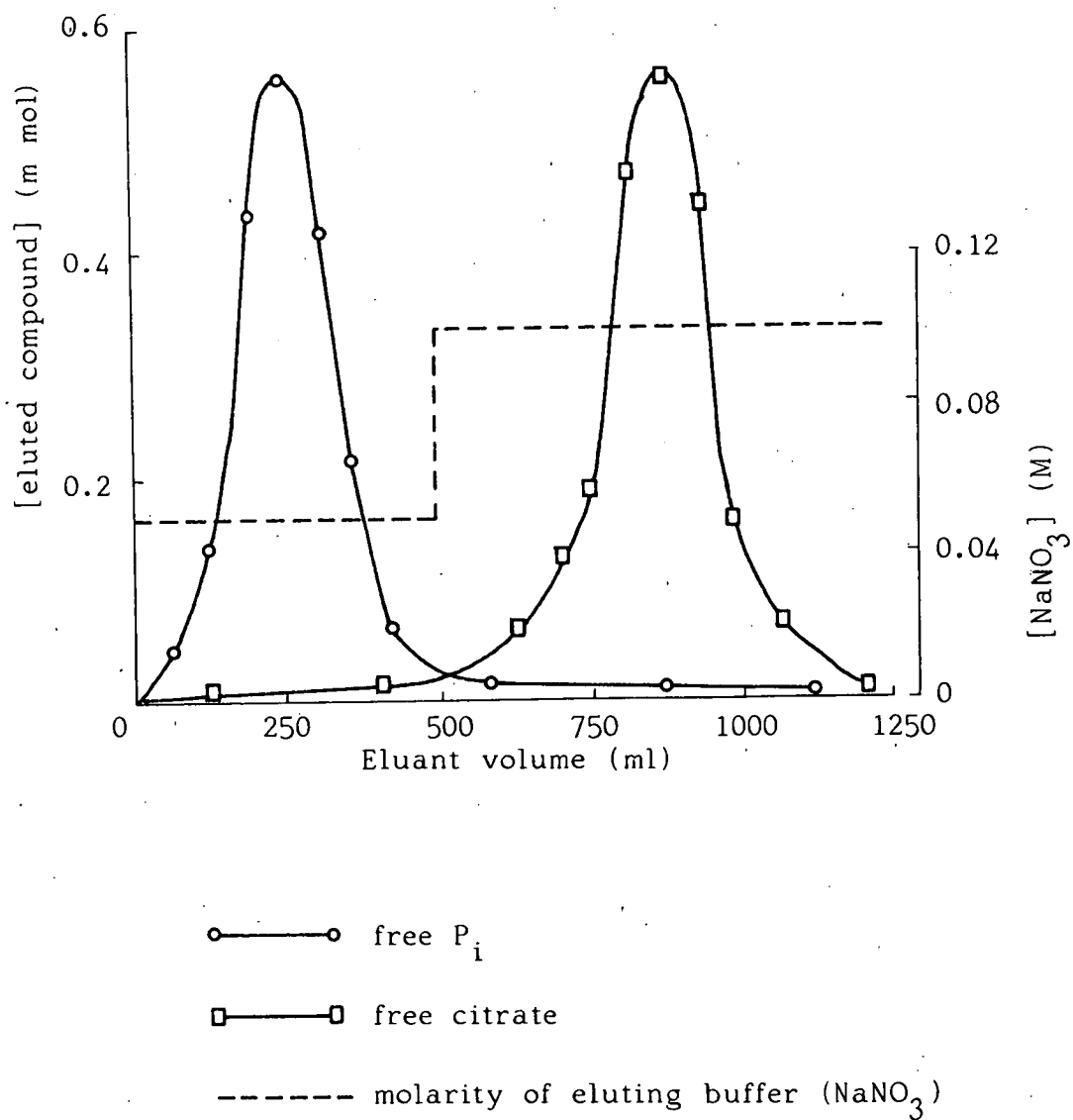


Figure 2.7 Attempt to separate components of the decalcified solution using an AG 1-X8 (NO_3) ion-exchange system.

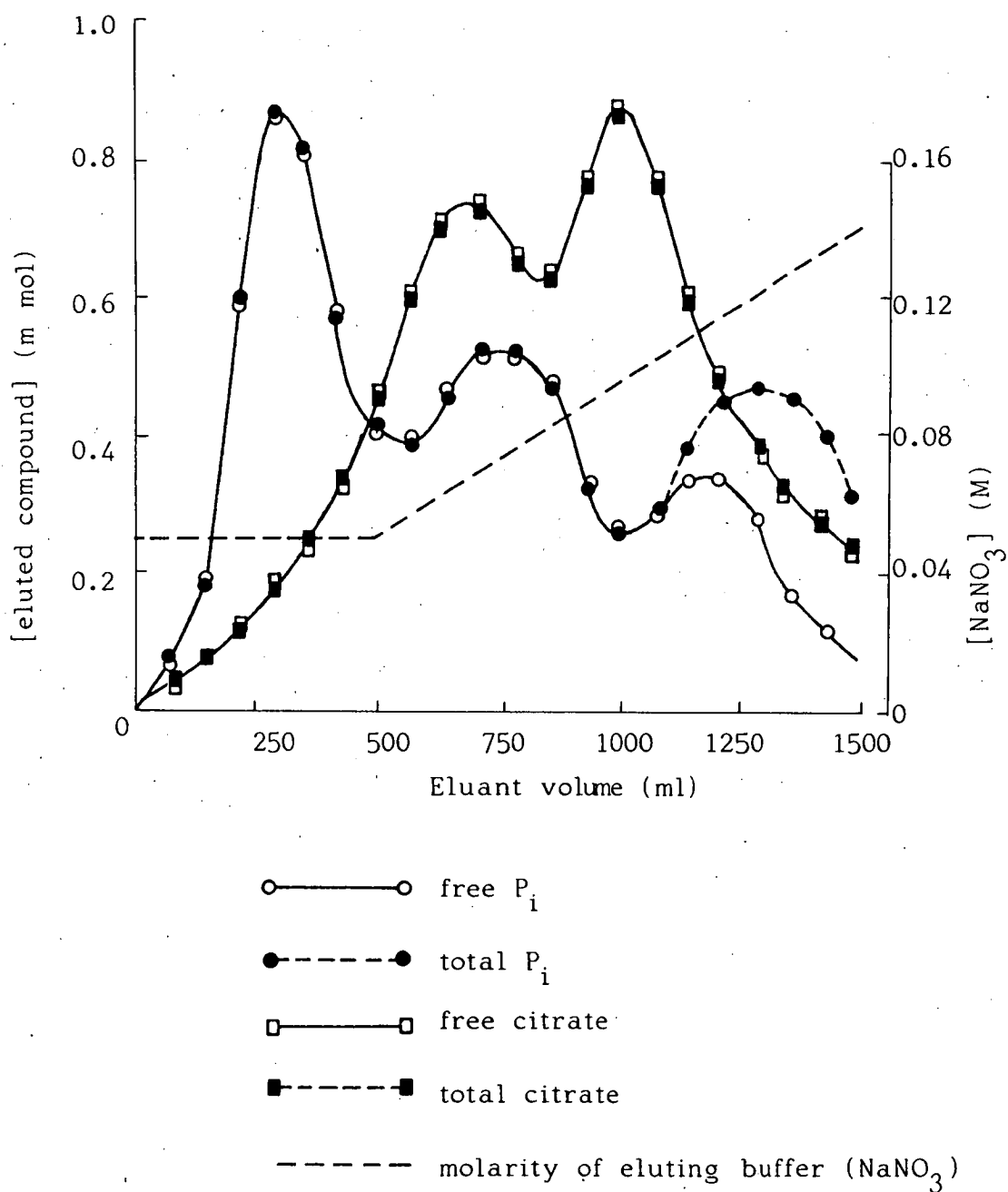
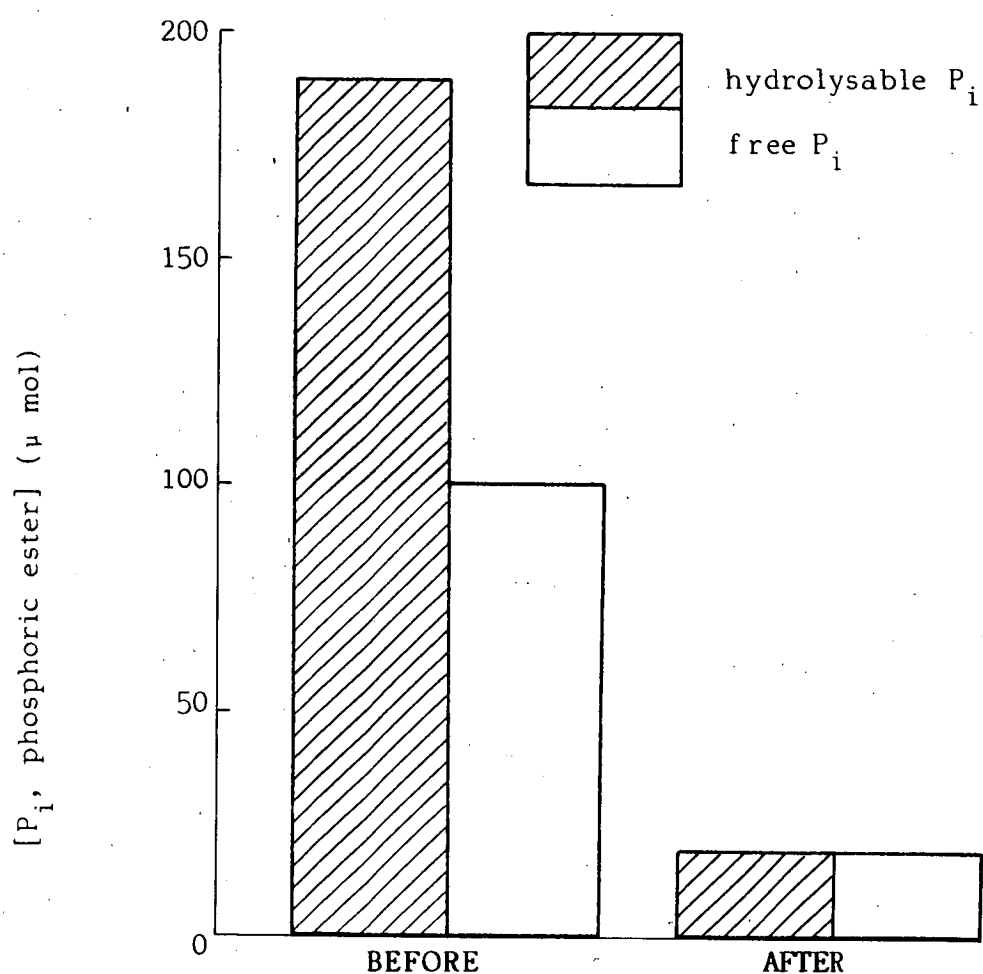


Figure 2.8 Loss of phosphoric ester product after treatment with activated charcoal.



200mg calcium precipitable material was dissolved by the addition of Dowex 50W $[H^+]$ and, after centrifugation, the supernatant was adjusted to a pH of 7 before treatment with activated charcoal. Given are the amounts of P_i and phosphoric ester in the supernatant.

Table 2.1 Decolouration of a quinone solution by various adsorbents.

ADSORBENT*	% DECOLOURATION [†]
Norit A	
(activated carbon)	93 [±] 6
Fuller's Earth	11 [±] 4
Florisil	
(60-100 mesh)	1 [±] 2
Celite 545 [‡]	-

*500g adsorbent added to 5ml of a 0.1% solution of the quinone. The solution was previously adjusted to a pH of 10, then neutralised.

[†]Values given are means \pm 1S.D.

[‡]Solution darkened by treatment

These difficulties, combined with the fact that the alternative method to remove the protecting group involved the use of platinum oxide, led to the more intense investigation of the use of 2-cyanoethyl phosphate (CEP) in the preparation of phosphocitrate. This reagent appeared to be particularly useful due to its easy preparation from $[^{32}\text{P}]\text{-P}_i$ which might enable ready $[^{32}\text{P}]$ -labelling of phosphocitrate.

2.4.1.2 Synthesis using 2-cyanoethyl phosphate

2.4.1.2.1 Preparation of crude phosphocitrate

The barium salt of 2-cyanoethyl phosphate was prepared from phosphorus oxychloride and 3-hydroxy propionitrile (hydracrylonitrile) as outlined by Tener (1961). Twenty-six grams of the compound was treated with AG 50W-X8 (H^+ ; 200ml resin bed volume) to remove the barium and the eluant concentrated by rotary evaporation.

The resulting oil was dissolved in 500ml of anhydrous pyridine before the addition of a two-fold excess of triethyl citrate (TEC) (44.0g). The mixture was evaporated at 30°C in vacuo to an oil, pyridine (800ml) was then added and the solution again evaporated. The process was repeated twice more before the final addition of 400ml of pyridine and 98g of dicyclohexylcarbodi-imide (DCC). The reaction flask was stoppered and allowed to stand at room temperature for 6 days with occasional stirring. At the end of this period, pyridine was evaporated off, 500ml of water added to decompose unreacted DCC, and the mixture stirred for 1h. The water was evaporated off, a further 500ml of water added

and the mixture stirred, and filtered. The residue was then stirred with a further 100ml of water to ensure complete recovery of the coupled product from the solid. The combined filtrate and washings were evaporated to give a golden brown oil.

The use of a two-fold excess of TEC results, after hydrolysis, in a high proportion of citrate in the mixture which will bind to ion-exchange resins necessitating use of adequate amounts of resin to bind the anions. Clearly if citrate could be removed, a smaller quantity of resin could be used, increasing the efficiency of the ion-exchange purification.

TEC is uncharged so it does not bind to AG 1-X8 resin indicating that if the unhydrolysed mixture was poured down a column of AG 1-X8, TEC might pass unhindered through the column whereas anionic species such as CEP and the coupled product (TEC-CEP) might bind and be recoverable by elution with the appropriate buffer. The advantage of purification at this stage of the synthesis is that since non-binding TEC is present rather than citrate, sufficient resin to bind only CEP and CEP-TEC need be used to afford resolution of the reaction components.

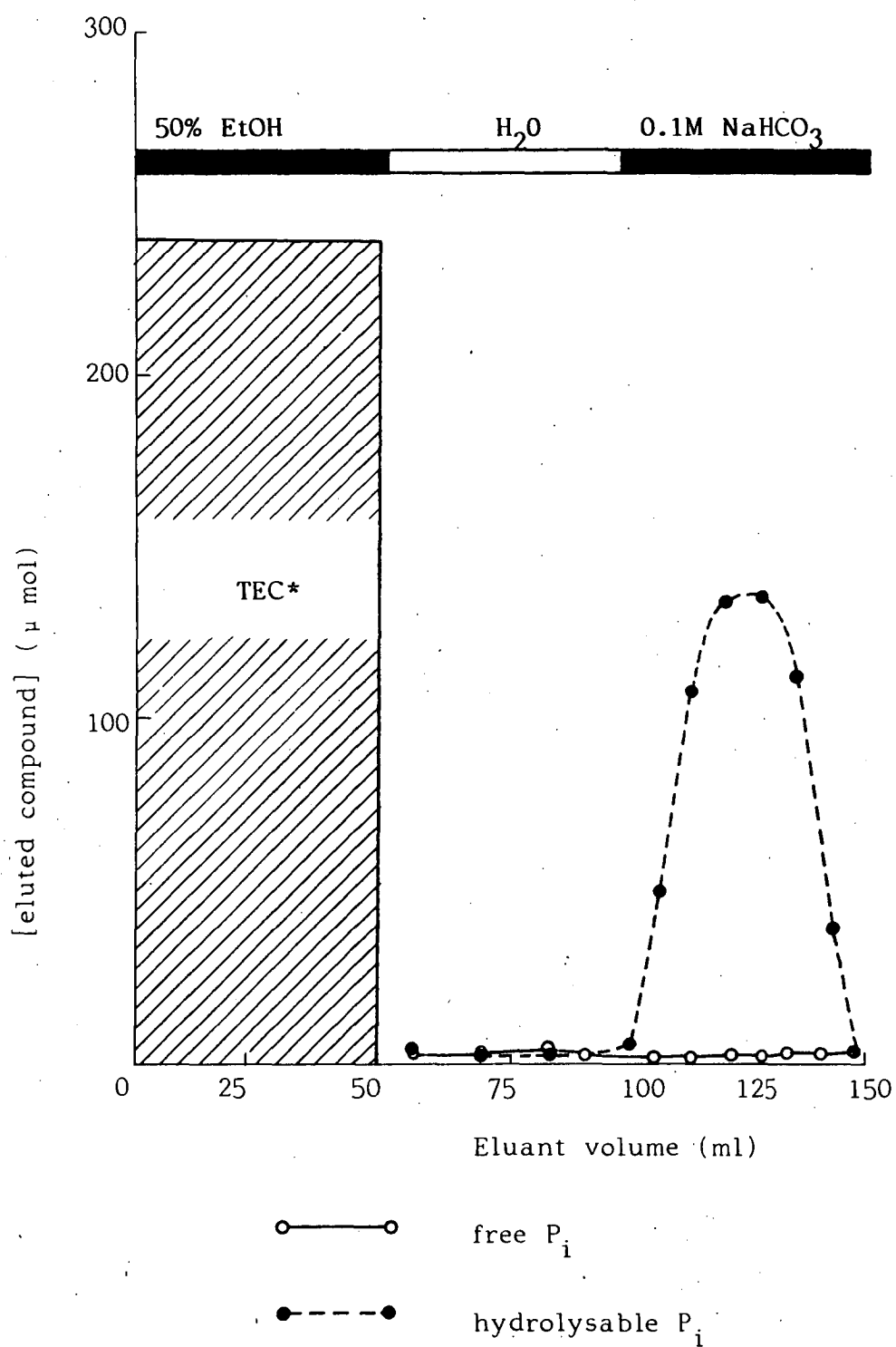
In a preliminary experiment, 200mg of the oil was dissolved in about 2ml of 50% EtOH adjusted to a pH of 7, and the solution was applied to a 1.5 x 5cm (AG 1-X8 (HCO_3^-)) column. The column was washed successively with 50ml each of 50% EtOH, water and 1.0M NaHCO_3 . The eluant was collected in 5ml fractions which were subsequently hydrolysed either in mild alkaline conditions to convert TEC to

citrate, or at 100°C for 4h in 0.1N H₂SO₄ for phosphate analysis. The results of such a column purification are given in Figure 2.9, indicating that unreacted TEC does indeed continue to wash through the column in 50% EtOH. No phosphate containing compounds were detected at this point, but were detected after elution with 0.1M NaHCO₃ suggesting that a separation of TEC from the desired coupled product was possible.

Consequently the pilot method was scaled up; the total amount of oil obtained after coupling (ca. 60g) was dissolved in a minimum of 50% EtOH (ca. 75ml), adjusted to a pH of 7.0 and applied to a 3.0 x 40cm column of AG 1-X8 (HCO₃⁻). Unreacted TEC was removed with 70ml of 50% EtOH (pH 7) at a flow rate of 1-2ml/min. In preliminary experiments, 0.27M NaHCO₃ was found to elute citrate and CEP, therefore a more concentrated buffer (0.45M) was used to ensure efficient elution of all material bound to the AG 1-X8 column. Buffer (2-2.5 L of 0.45M NaHCO₃) was passed through the column and the eluant subsequently decarbonated with AG 50W-X8 (H⁺ form). After filtration, the filtrate was evaporated to dryness either by lyophilisation or by rotary evaporation at 30°C in vacuo. This procedure resulted in 19.2g of a golden oil (30-35% yield).

Tener (1961) has reported that, unlike free CEP, bound CEP is quite labile and that the cyanoethyl group can be readily hydrolysed in mild alkali at 50°C in 2 min giving the required phosphoric ester. Consequently, the coupled product isolated by ion-exchange chromatography was treated in a similar way with the exception that calcium was

Figure 2.9 Separation of TEC from the coupled intermediate using an AG 1-X8 (HCO_3^-) column.



*Expressed as μmol of citrate released after alkaline hydrolysis.

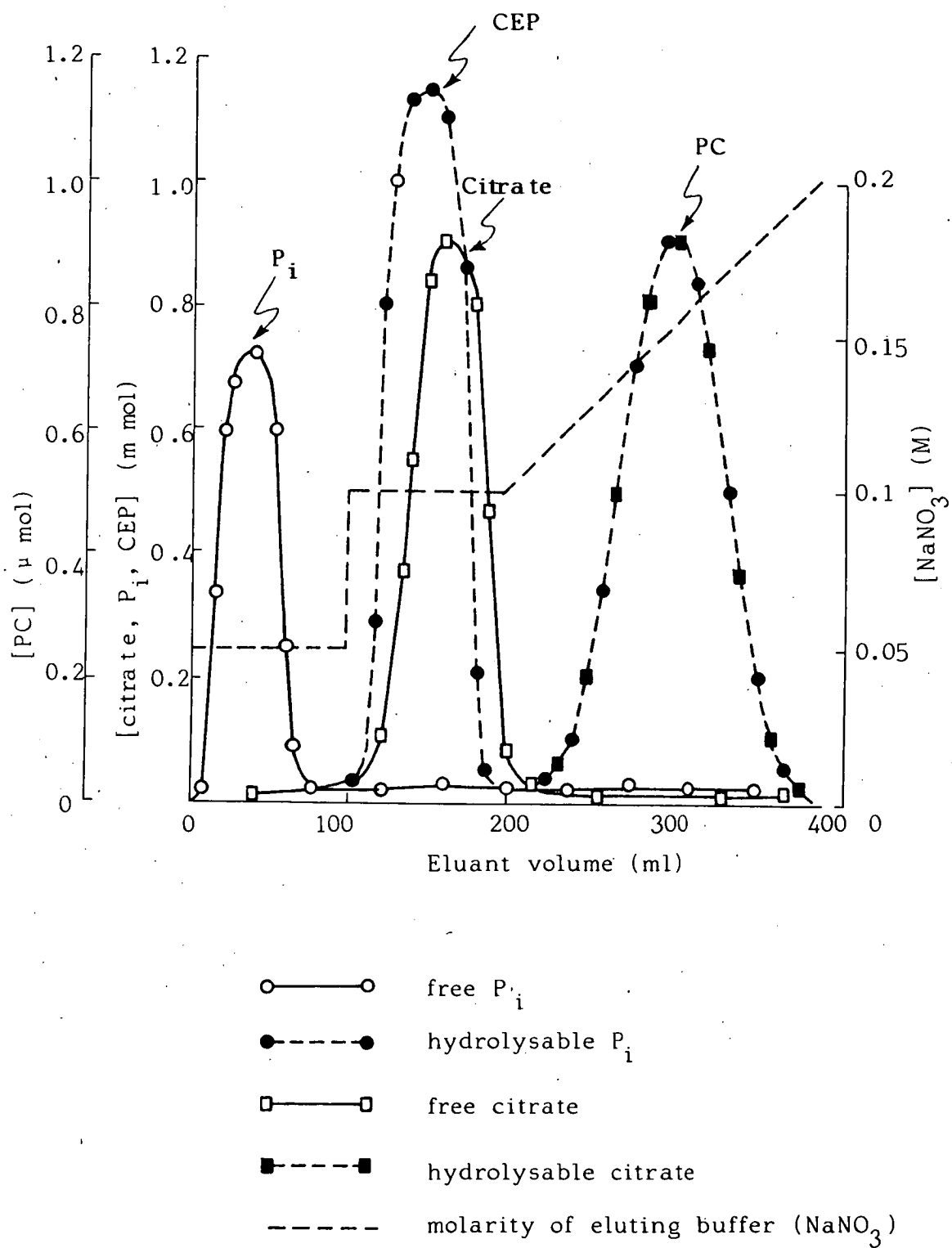
included in the hydrolysis mixture in order to precipitate phosphocitrate as it formed. This latter approach was based on the work of Meyer et al. (1959). Accordingly, 5.4g of the coupled product was stirred with 1000ml of 0.1N NaOH and 7.0g of CaCl_2 at 50°C for 5-10 min before pouring into a chilled beaker. The beaker and its contents were cooled to 4°C and left overnight. After adjusting to a pH of 8.5 the mixture was lyophilised, 100-150ml of water added, and the mixture left at 4°C for 1-2h. The solid was collected by centrifugation (800-1000g), washed with absolute EtOH, acetone and ether respectively, giving 4.5g of a white solid.

2.4.1.2.2 Ion-exchange chromatographic purification of phosphocitrate

In order to improve the ion-exchange separation of citrate and phosphocitrate over the previously described system, which used a gradient of HCl (Meyer et al., 1959), and to permit separation at pH 7, the use of an AG 1-X8 (NO_3^-) system was investigated. Nitrate - a strongly binding anion - was foreseen as eluting citrate and PC more readily, and thereby enabling better resolution of these anions.

In order to apply this system to the purification of phosphocitrate, 500mg of the impure salt was dissolved in 10ml water with the aid of 5ml of AG 50W-X8 (H^+ form). The mixture was applied to a 1.5 x 5cm column of AG 50W-X8 and the eluant collected and adjusted to a pH of 5-7 before loading into a 1.5 x 10cm column of AG 1-X8 (NO_3^-) at a flow rate of $1\text{-}2\text{ml min}^{-1}$. As shown in Figure 2.10, the batch-wise elution of phosphate, citrate and CEP and subsequent application of a linear gradient of NaNO_3 (0.1M to 0.2M)

Figure 2.10 Purification of PC from citrate, CEP and P_i using AG 1-X8 (NO_3^-) chromatography.



eluted a product which, when hydrolysed, contained P_i and citrate in a 1:1 molar ratio - presumably phosphocitrate. Although the separation of citrate and P_i from PC was complete, it was found that the isolation of PC from the buffer was difficult due to the need to form an insoluble salt. Since $Ca(NO_3)_2$ is soluble in EtOH the calcium salt of PC was precipitated after adding 2 volumes of EtOH to the relevant tubes. These conditions, however, also led to the precipitation of $NaNO_3$. Instead of persisting with this method which would ultimately have yielded a calcium salt contaminated with $NaNO_3$, an alternative method was sought because the sodium salt is more useful for routine work.

Another anion with almost the same binding affinity as nitrate is bicarbonate which has the further characteristic of decomposing in acid to give carbon dioxide. Consequently if sodium bicarbonate buffers are used then the addition of the resin AG 50W-X8 (H^+) would not only bind sodium ions but, by the subsequent release of protons into solution, decompose bicarbonate. The pH during this process falls to 4-5 so if this procedure was performed at $4^\circ C$ PC hydrolysis should be minimal.

An important requirement for any chromatographic system is that it operates at neutral pH. In this respect 0.5M $NaHCO_3$ stock solution gassed with CO_2 for 10-15 min became acidified to a pH of 7-7.2. This process did not significantly affect the bicarbonate content of the stock solution as indicated by conductimetric measurements on solutions of various HCO_3^- concentrations, before and after

bubbling with CO_2 (Figure 2.11). In order to determine the concentration of bicarbonate needed for batch-wise resolution, preliminary work used a linear gradient to elute P_i , citrate and CEP from an AG 1-X8 (HCO_3^-) column and established that 0.27M NaHCO_3 was the minimum concentration required to batch-elute these anions. Treatment of 300mg of the impure salt with Dowex 50W-X8 (H^+ form) gave a Ca^{++} -free solution which was adjusted to a pH of 7 and loaded onto a 1.5 x 10cm column of AG 1-X8 (HCO_3^-) resin at a flow rate of 1-2ml/min. Batch-wise elution with 0.27M NaHCO_3 removed P_i , citrate and CEP, and PC was eluted using 0.45M NaHCO_3 .

Scaling up this procedure involved passing 4.5g of the dissolved impure salt through a column of AG 50W-X8 (40ml resin bed volume) and adjusting the eluant to a pH of 7 before loading onto a 3 x 15cm column of AG 1-X8 (HCO_3^-). Phosphate, citrate and CEP were eluted with 2-2.5L of 0.27M NaHCO_3 , and PC was then recovered by elution with 0.45M NaHCO_3 , collected in 300 fractions of 10ml volume (Figure 2.12). The presence of PC was detected by analysis of P_i and citrate before and after hydrolysis. Contents of tubes containing PC were pooled, treated with AG 50W-X8 (H^+) in the cold, the pH of the filtrate adjusted to 7 with diluted NaOH, and the solution lyophilised to give a white powder. The powder was dissolved in a small amount of water (20-50ml) and decarbonated again to ensure complete removal of excess salt. Lyophilisation gave sodium phosphocitrate which was stored in a desiccator at -20°C . Analysis revealed the sample contained <1% inorganic phosphate and after 3

Figure 2.11 Conductivity of NaHCO_3 solutions before and after gassing with CO_2 .

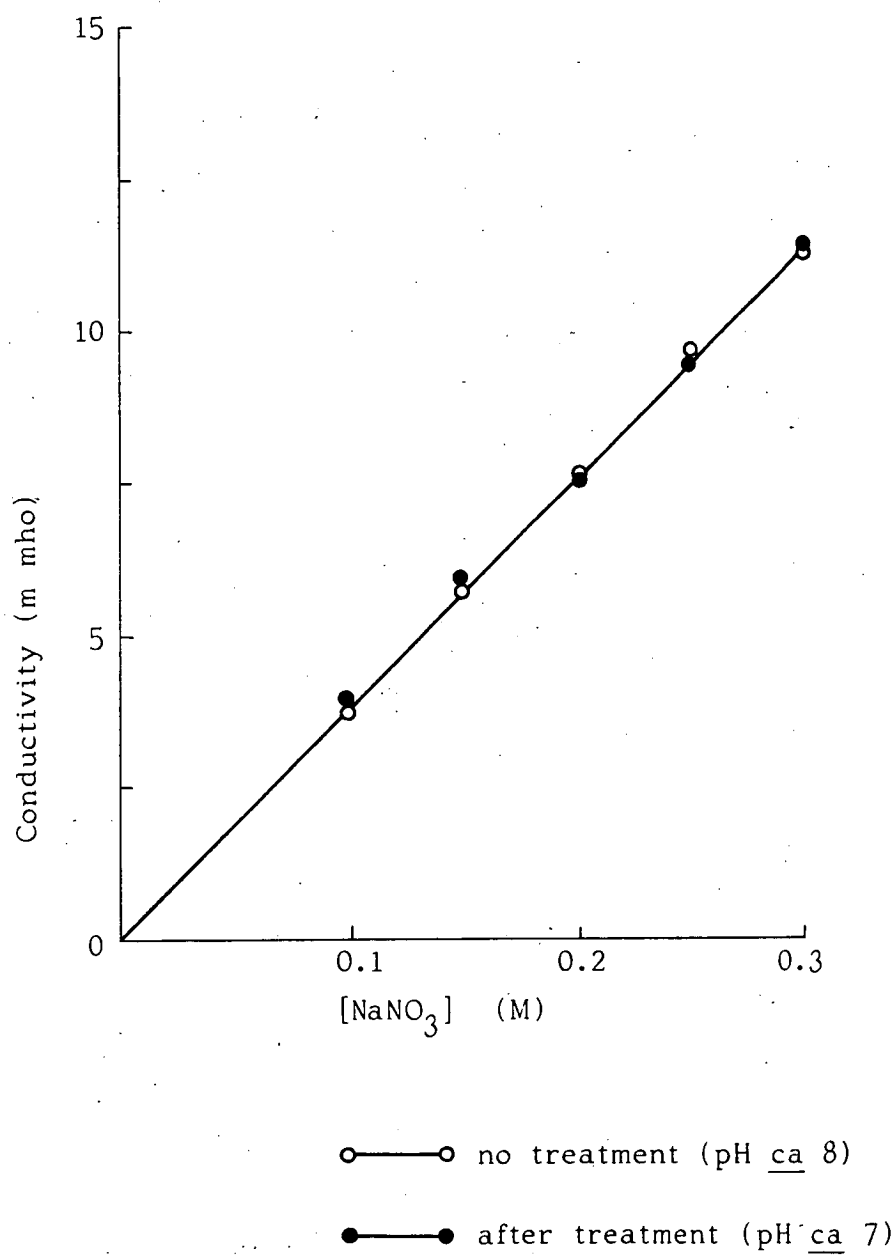
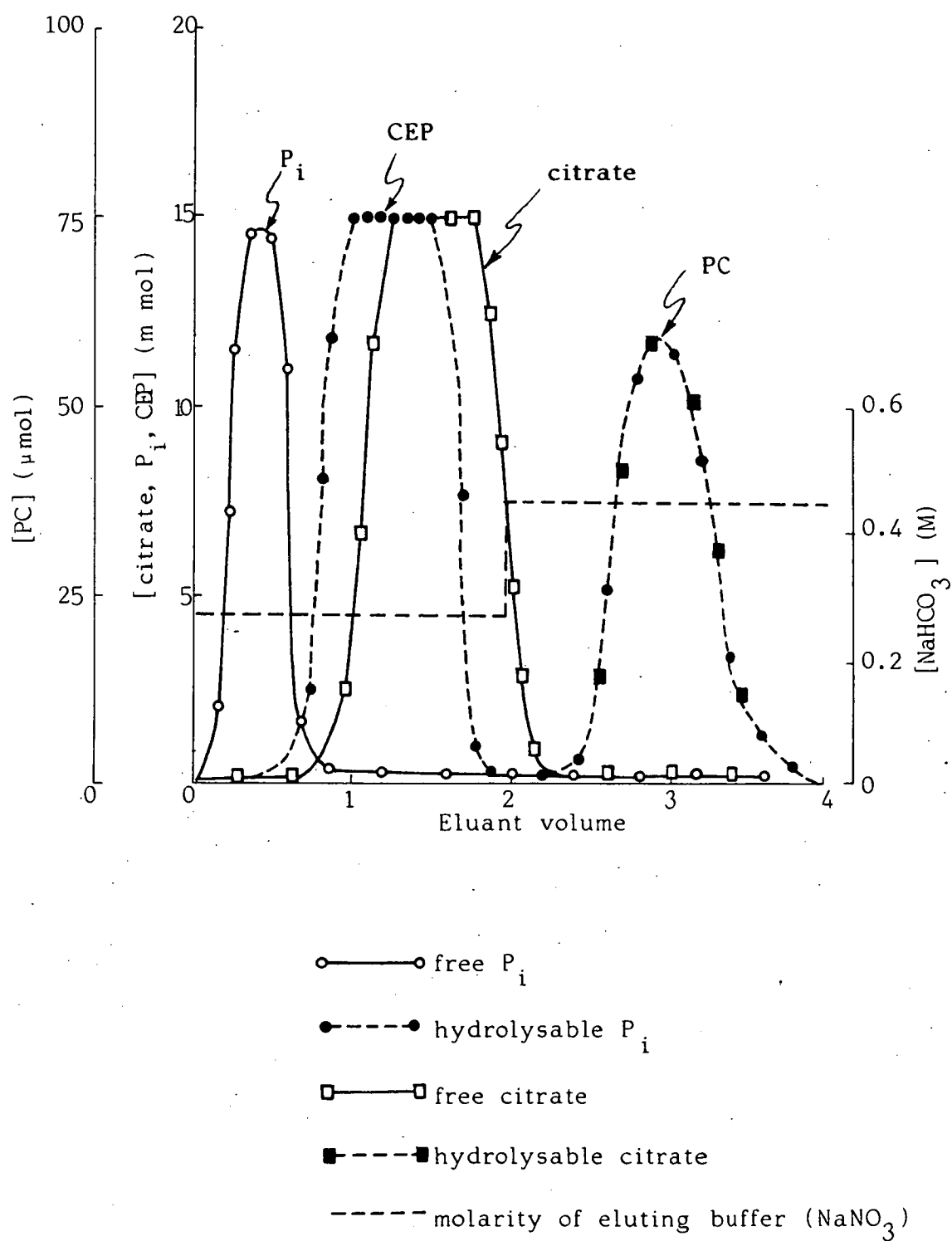


Figure 2.12 Purification of PC on an AG 1-X8 (HCO_3^-) resin column using sodium bicarbonate buffers.



months there was no evidence of decomposition. Determination of the total phosphate content of the solid enabled calculation of the molecular weight of the salt. This was found to be 445gmol^{-1} corresponding to a molecular formula of $\text{Na}_5\cdot\text{PC}\cdot\text{ca. } 4\text{H}_2\text{O}$.

2.4.2 SYNTHESIS OF [^{32}P]-LABELLED PHOSPHOCITRATE

Barium [^{32}P]-cyanoethyl phosphate is available from the Radiochemical Centre, Buckinghamshire, Amersham, England; but in this instance labelled reagent was prepared from 120mg of [^{32}P]-phosphoric acid as described by Tener (1961). Barium was removed by treatment with AG 50W-X8 (pyridinium form; 7ml resin bed volume) and the eluant, after the addition of 5ml of pyridine, was evaporated to dryness in vacuo at 30°C . A large excess of TEC was added (200mg) together with 10ml of pyridine. The solution was evaporated to an oil, a further 10ml of pyridine added, and the solution again evaporated. This procedure was repeated twice before the addition of 5.0ml of pyridine and 800mg of DCC. The flask was set aside for 3 days before evaporation and the addition of 5ml of water to decompose surplus di-imide. The mixture was stirred for 1h, evaporated, and another 5ml of H_2O added. Following filtration the residue was washed and the combined filtrate and washings evaporated to give a golden oil.

The oil obtained was treated with 140ml of 0.1M NaOH and 600mg of CaCl_2 at 50°C for 7 min before the suspension was rapidly cooled and allowed to stand at 4°C overnight. Fifteen mg of unlabelled PC was added, the mixture adjusted

to a pH of 8.5, lyophilised, the residue taken up in 10ml of water, centrifuged, and the precipitate washed as before. After treatment with AG 50W-X8 (8ml resin bed volume), the material was loaded onto a 1.5 x 10cm column of AG 1-X8 (HCO_3^-). This column was treated in parallel with another column loaded only with unlabelled PC and both columns were then washed, each with 400ml 0.27M NaHCO_3 . A volume (1L) of both 0.27M and 0.5M NaHCO_3 buffer were used to provide a linear gradient to both columns simultaneously and 300 fractions of 4ml volume were collected. Either the radioactivity of the tube contents was determined, or P_i was analysed after hydrolysis. Relevant tube contents were pooled and decarbonated as before to give sodium phosphocitrate of specific activity $200\mu\text{Ci}/\text{m mol}$.

2.4.3 CHARACTERISATION OF PC

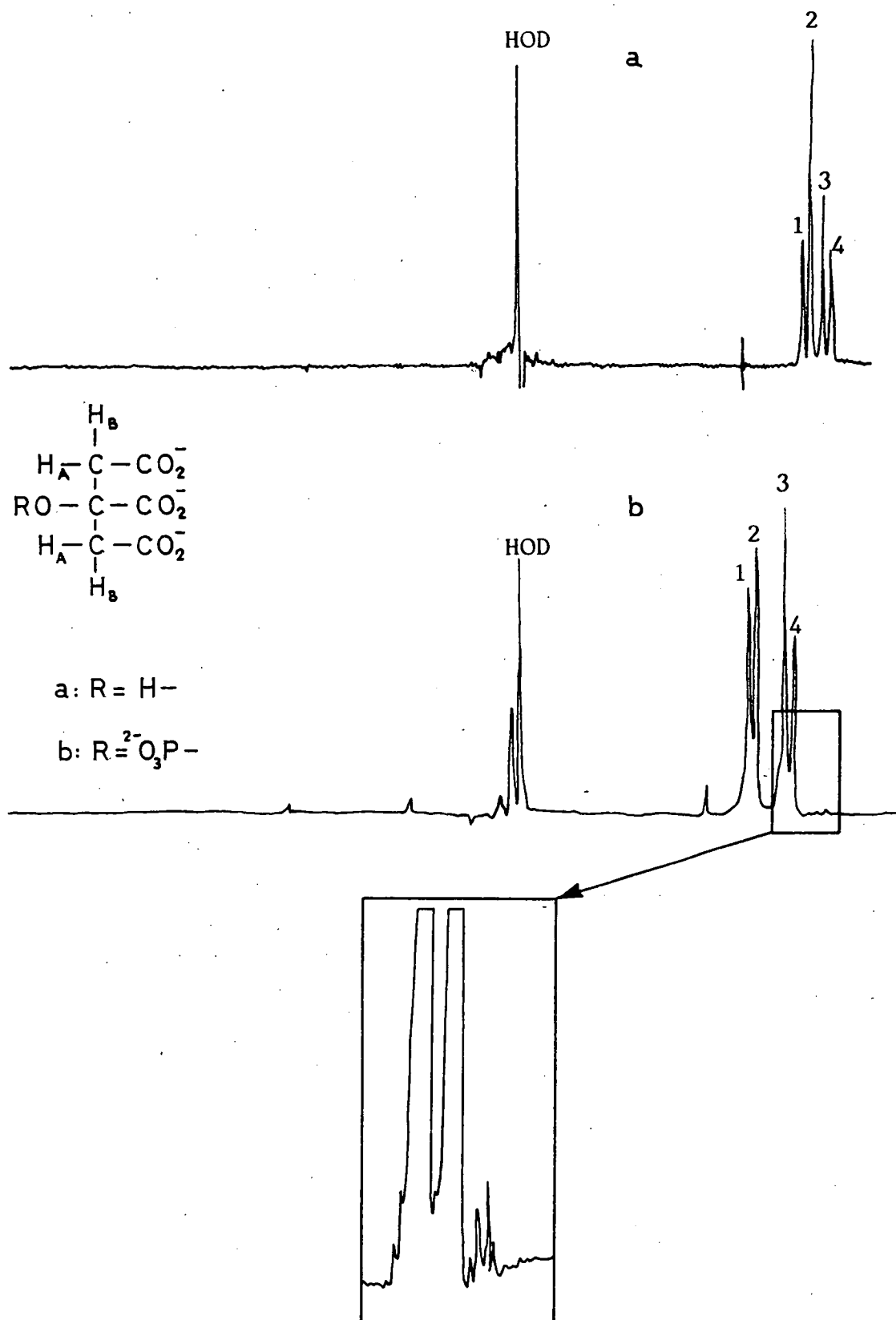
2.4.3.1 Spectroscopic

NMR spectra were recorded at the National NMR Centre, Canberra, Australia (^1H NMR at 270M Hz, ^{13}C NMR at 67.889M Hz) or at Oxford University, U.K. (^{31}P NMR at 7kM Hz).

2.4.3.1.1 ^1H NMR spectroscopy

^1H NMR spectra of both citrate and phosphocitrate were recorded for comparative purposes, at a pH of 7.3 and ambient temperature. The spectra are shown in Figure 2.13 and both display a characteristic AB type spectra (Dyer, 1965). The lack of any other peaks in the spectrum of PC attests to the purity of PC. However, if the area up-field of the PC peaks (representing the area where citrate protons resonate) is amplified, peaks due to citrate can

Figure 2.13 ^1H NMR spectra of: (a) citrate, and (b) phosphocitrate.



be recognised (Figure 2.13); this indicates that the level of citrate present in the sample is <1%.

2.4.3.1.2 ^{13}C NMR spectroscopy

The ^{13}C NMR spectra of citrate and PC were determined with sample conditions 25°C and pH 7.1. Coupling of the ^{31}P nucleus to the ^{13}C nucleus can be seen to give rise to characteristic splitting patterns in the spectra of phosphocitrate (Figure 2.14). The lack of any other peaks apart from the ^{13}C -PC resonances again attests to the purity of synthetic PC.

2.4.3.1.3 ^{31}P NMR spectroscopy

^{31}P NMR spectroscopy at 7kM Hz indicated that, relative to 88% H_3PO_4 , PC resonates at 2.1, 1.1 and 0.2 ppm at pH's of 5, 7 and 8.5 respectively. Notably the peaks were singlets. If the reference was changed to methylene diphosphonate, then the absence of P_i is demonstrable; this is shown in Figure 2.15.

The pH-dependence of the PC ^{31}P -resonance is shown in Figure 2.16, over the pH range 2.5 to 11, and is compared with a variety of other organic phosphates (Ogawa *et al.*, 1978). Due to the number of dissociating groups in PC and the lack of detail in the pH-dependence curve, the pK's could not be determined.

2.4.3.2 Lability

2.4.3.2.1 Chemical lability

The lability of a 1mg/ml solution of PC was determined over a wide range of conditions, as illustrated

Figure 2.14 ^{13}C NMR spectra of: (a) phosphocitrate, and (b) citrate.

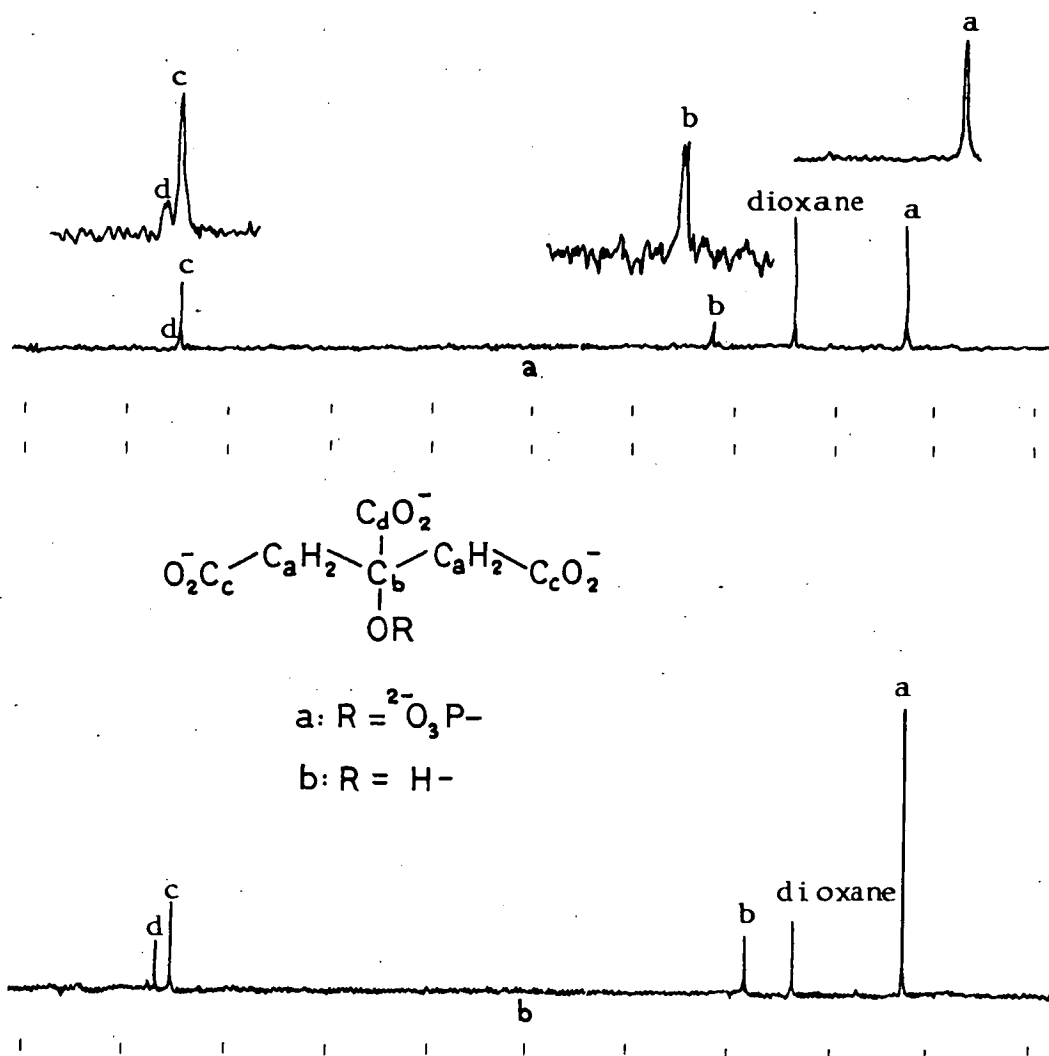
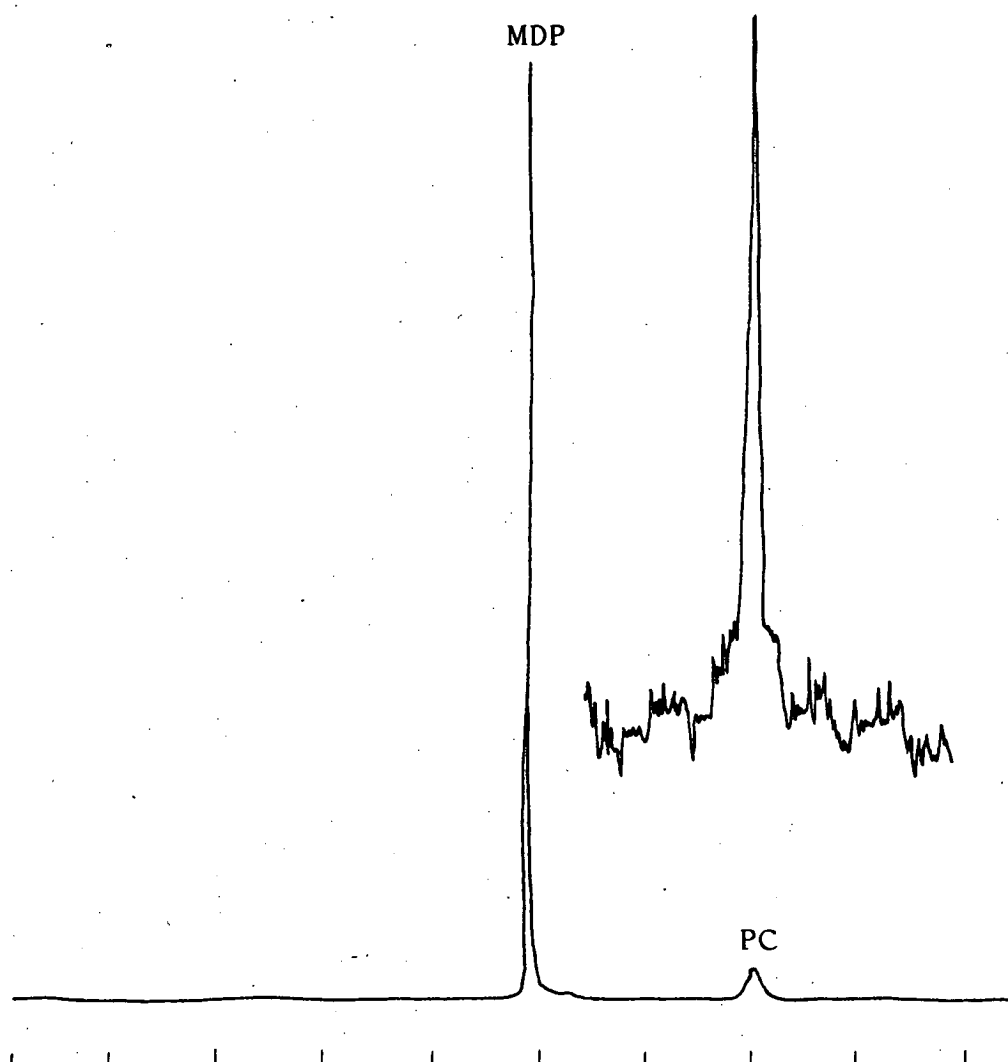


Figure 2.15 ^{31}P NMR spectrum of phosphocitrate*.

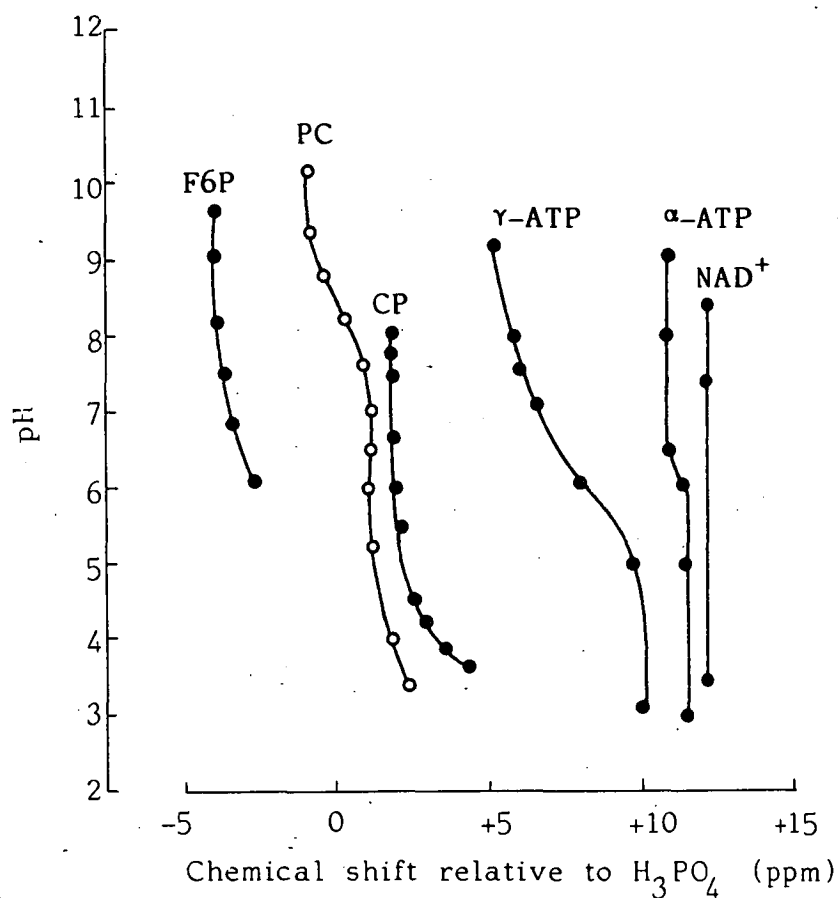


MDP = methylene diphosphonate

PC = phosphocitrate

*Determined at pH 7, ambient temperature.

Figure 2.16 pH-dependent chemical shift behaviour of PC compared with other phosphoric esters*



F6P	fructose 6-phosphate
PC	phosphocitrate
CP	creatine phosphate
γ-ATP	γ-P in ATP
α-ATP	α-P in ATP

* PC data has been superimposed on data obtained for other phosphoric esters as determined by Ogawa *et al.* (1978).

in Figure 2.17. Notably PC appeared to be more labile to heat than any particular pH; PC decomposed within 4h at 100 °C whether conditions were acid or alkaline. In contrast, at 0 °C solutions were stable indefinitely. At room temperature, PC solutions showed intermediate stability, decomposition occurring slowly over a period of days.

2.4.3.2.2 Enzymatic lability

To determine whether PC was degraded by phosphatase activity, the release of citrate was monitored during an incubation with either acid or alkaline phosphatase. Using 0.71 U of acid phosphatase, 0.093 μ mol PC was incubated at 37 °C and a pH of 4.8: using 4.4 U of alkaline phosphatase, 0.35 μ mol PC was incubated at 37 °C and a pH of 10.4. The results are given in Figure 2.18 and indicate PC is completely hydrolysed within 15 min with either phosphatase. From the rates of hydrolysis under these standard conditions, it was estimated that the enzymatic lysis of PC was 3% or 2% that of *p*-nitrophenyl phosphate with acid or alkaline phosphatase respectively.

2.5 DISCUSSION

The data presented here indicate that PC can be prepared from CEP and TEC. This method offers several advantages over that using dibenzyl chlorophosphate since the yield is higher, specialised equipment is not necessary and, most importantly, the CEP method can be readily applied to the synthesis of [32 P]-PC. Using *o*-phenylene chlorophosphate, the initial yield was found to be superior to that using

Figure 2.17 Stability of PC under various conditions.

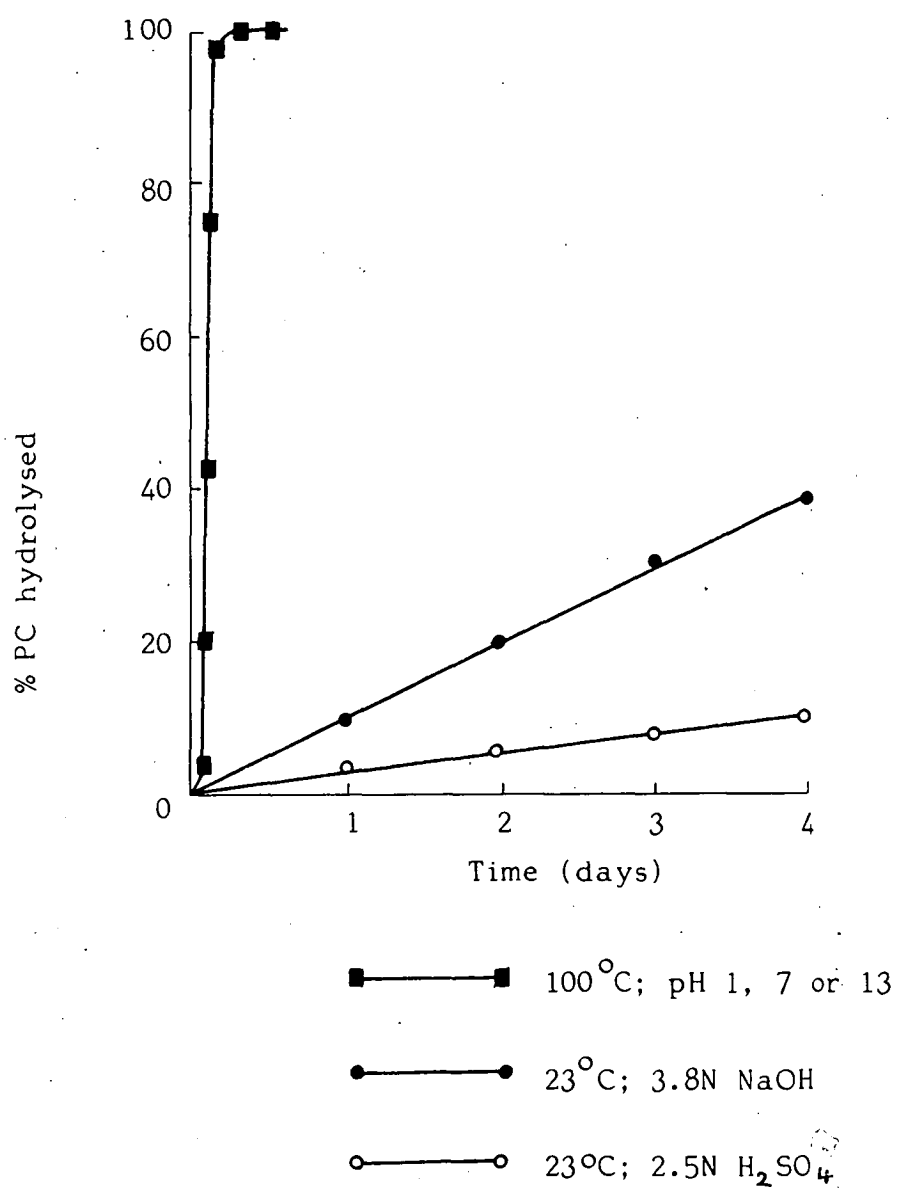
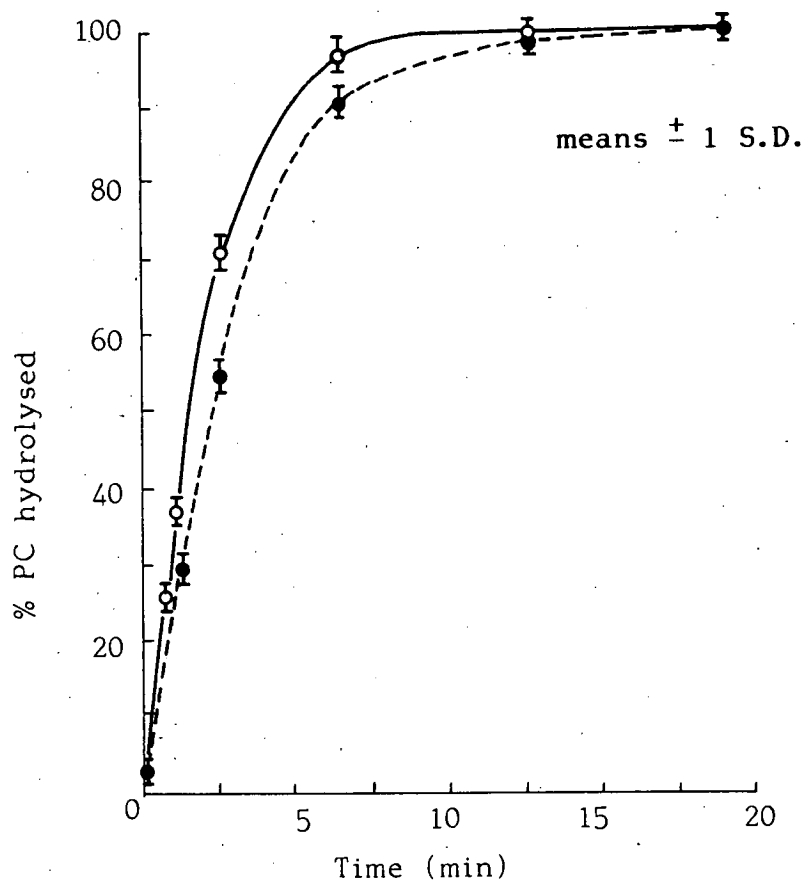


Figure 2.18 The degradation of PC by either acid or alkaline phosphatase activity.



○ — ○ alkaline phosphatase

● - - ● acid phosphatase

CEP, reflecting its phosphorylating power. However, oxidation apparently liberates o-benzoquinone (Khwaja et al., 1970), a reactive species which not only forms polymeric material but disrupts the binding and resolution of ion-exchange columns. The alternative is to catalytically hydrogenate the group using platinum oxide, an expensive procedure. Even if this is done, it is synthetically difficult to introduce a label into PC since either $[^{32}\text{P}]\text{-PCl}_5$ must be used to synthesize the phosphorylating reagent, or $[^{14}\text{C}]\text{-citrate}$ to prepare the triethyl ester for PC synthesis. Consequently, despite its lower yield, the ease and relative cost of preparing $[^{32}\text{P}]\text{-CEP}$ from $[^{32}\text{P}]\text{-P}_i$ makes it the reagent of choice for the synthesis of $[^{32}\text{P}]\text{-PC}$.

Proof that the compound prepared by the CEP method is indeed PC, in which the phosphate group is esterified to the tertiary hydroxyl group rather than one of the carboxylic acid moieties - citryl phosphate - rests on the following observations:

1. The use of triethyl citrate (>99% purity) ensures that it is only the tertiary hydroxyl that is phosphorylated rather than any of the "blocked" carboxyl groups.

2. Acyl phosphates are significantly more unstable than phosphoric esters. In fact, citryl 1-phosphate has been prepared (Gulyi et al., 1963, 1965; Walsh and Spector, 1968, 1969; Walsh, 1970) and has been found to be highly labile: it can only be stored for 2 days in a desiccator in vacuo and at 20°C will completely decompose in 1h in an aqueous solution at pH 8.5-9. In contrast, the data presented here indicate that PC can be stored, desiccated, for at least 6 months at -20°C with no discernible change. Further, no

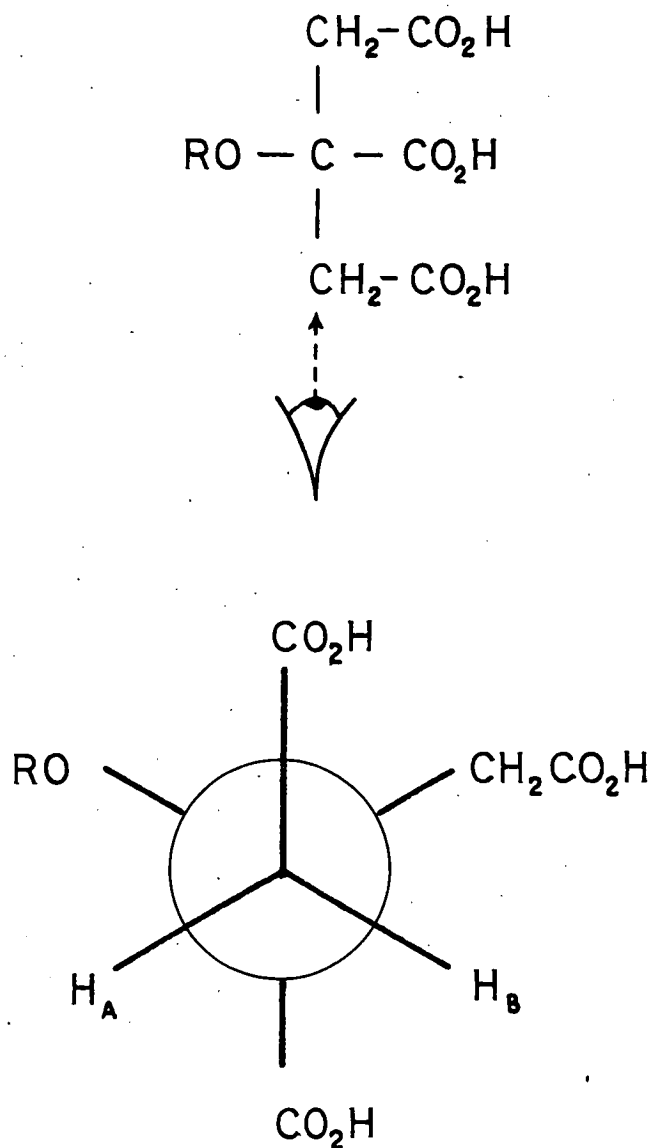
significant changes occur when PC is kept in alkaline solution at room temperature for many hours.

3. Spectroscopic evidence unambiguously characterised PC. As shown in Figure 2.19, the methylene protons of both citrate and PC are never equivalent in any conformation so that the unequal protons interact causing splitting and giving rise to the typical AB pattern. Particularly characteristic in the ^1H NMR spectrum of PC is the downfield shift of the AB pattern relative to citrate due to the presence of the electron withdrawing phosphate group which deshields the H_A and H_B protons. Only citryl 3-phosphate in which phosphate is esterified to the central carboxyl group could give rise to the AB pattern since 1 or 6 substitution (citryl 1-phosphate) would result in an ABXY system (Figure 2.20).

The ^1H NMR spectra of citrate and PC allow various characterising parameters to be calculated (Dyer, 1965), including the chemical shifts (δ) for the hypothetical non-interacting H_A and H_B , the differences in chemical shifts between H_A and H_B (δ_{AB}), and the H_A - H_B coupling constants (J_{AB}). These data are given in Table 2.2

Spectroscopic proof that citryl 3-phosphate had not been prepared is given by analysis of the ^{13}C NMR spectra, although other considerations, for example lability, also rule out the possibility. The peaks in the ^{13}C NMR spectrum of citrate are assigned according to the published data of Johnson and Jankowski (1972). The signal from the methylene carbons (C_a) is enhanced due to the nuclear Overhauser effect. Significantly, all the peaks are singlets. In the ^{13}C NMR spectrum of PC, ^{31}P - ^{13}C coupling is expected since

Figure 2.19 Non-equivalence of the methylene protons in citric acid and phosphocitric acid



In no conformation can $H_A = H_B$: they are non-equivalent.

phosphocitric
acid:
citric acid:

$R = H_2O_3P-$
 $R = H-$

Figure 2.20 Expected ABXY system for the ^1H NMR spectrum of citryl 1-phosphate

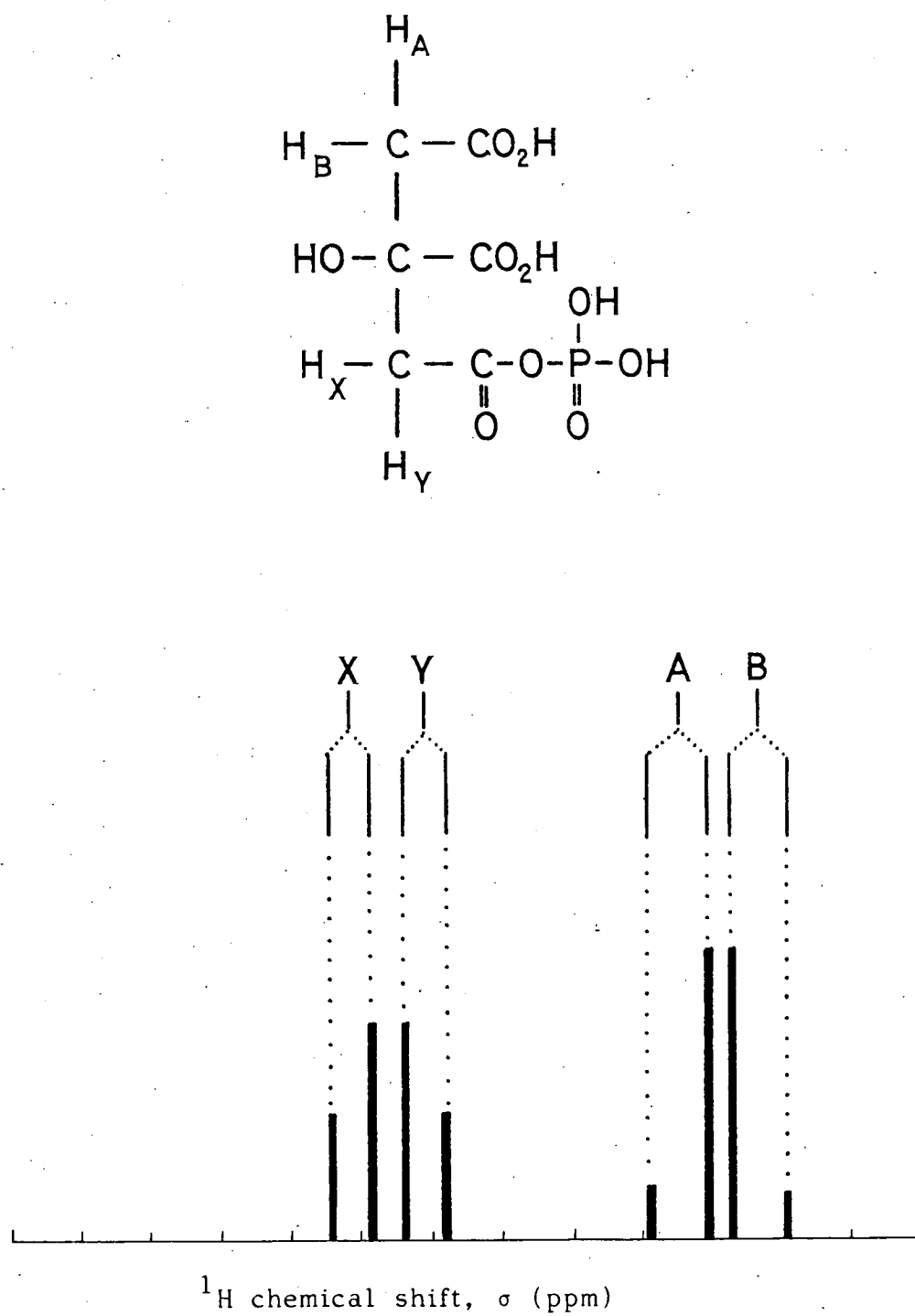


Table 2.2 ^1H NMR chemical shift (σ) data, coupling constants (J_{AB}) and differences in chemical shift between protons A and B (σ_{AB}) for citrate and PC*.

CITRATE					PHOSPHOCITRATE				
Peak	σ (ppm)	Peaks ⁺ σ	J_{AB} (Hz)	σ_{AB} (Hz)	Peak	σ (ppm)	Peaks ⁺ σ	J_{AB} (Hz)	σ_{AB} (Hz)
1	2.00	1.96	16	34	1	2.45	2.42	15.5	72
2	1.94	1.96			2	2.39	2.42		
3	1.86	1.83			3	2.17	2.15		
4	1.80	1.83			4	2.12	2.15		

*Determined at pH 7.3, ambient temperature.

⁺Value calculated for uninteracting H_A and H_B .

phosphorus-31 has a nuclear spin. Indeed, as shown in Figure 2.14, the central carboxyl carbon (C_d) and the central carbon (C_b) are split into doublets from which ^{31}P - ^{13}C coupling constants (J_{CP}) are calculable. These are given in Table 2.3, together with peak positions for citrate and PC.

Another characteristic feature of the ^{13}C NMR spectrum of PC is the nature of the chemical shifts relative to citrate. In fact it is these that indicate where citrate is phosphorylated. Attachment of a phosphate group is known to cause a β -carbon downfield shift and a γ -carbon upfield shift (Mantsch and Smith, 1972), the latter being dependent on the stereochemistry of the molecule. Thus the γ -methylene carbons (C_a) are not significantly affected, whereas there is a shift ($\gamma_P = -3.2\text{Hz}$) with the other γ -carbon (C_d). The β -carbon (C_b) is shifted downfield as expected ($B_P = 6.1\text{Hz}$). The B_P -downfield and γ_P -upfield shifts are consistent only with the phosphate being esterified to the tertiary hydroxyl group. If phosphate were esterified to the peripheral carboxyl group - citryl 1-phosphate - a different pattern of shifted peaks would result. Further, with citryl 3-phosphate which is phosphorylated at the central carboxyl group, the shifts would be the reverse of those observed.

Although the ^{31}P NMR spectrum does not add to the structure proof given above, comparison with a range of other organic phosphates (Figure 2.16) indicates that the PC resonance is characteristic. The change in the ^{31}P -resonance with pH (Figure 2.16) does not enable the pKs's for phosphocitrate to be determined, presumably because the dissociations occur over a relatively small pH range.

Table 2.3 ^{13}C NMR chemical shift (σ) data for citrate and PC and ^{31}P - ^{13}C coupling constants (J_{CP}) for PC*

CARBON ATOM	CITRATE σ (ppm)	PHOSPHOCITRATE	
		σ (ppm)	J_{CP} (Hz)
C _a	-20.70	-20.98	$^3J_{\text{CP}} < \pm 2.5$
C _b	8.61	14.74	$^2J_{\text{CP}} 7.4$
C _c	112.71	111.68	$^4J_{\text{CP}} < \pm 3.0$
C _d	115.35	112.12	$^3J_{\text{CP}} 7.4$

*Determined at pH 7.1, 25°C.

In order that pure material was available for spectroscopic analysis and for reference purposes, ion-exchange procedures had to be developed due to certain faults associated with the previously reported systems. Thus the ion-exchange method reported here using sodium bicarbonate buffers offers several advantages:

1. Separation is complete giving pure PC uncontaminated with citrate or P_i enabling its direct use, without further purification, in calcifying systems.

2. The neutral operating conditions circumvent the possibility of artefact formation thus allowing the potential use of this system in biological investigations where quantification is a paramount consideration.

3. The use of sodium bicarbonate allows the isolation of sodium PC quite readily since decarbonation is effected simply by the addition of an acidic ion-exchanger.

Resins exchanged with other anions, for example nitrate, necessitate the ultimate isolation of PC as an insoluble salt. However, the nitrate based system does afford slightly better resolution and therefore could be of use to separate [^{32}P]-compounds when recovery of compounds from eluants is not required. For most purposes it would appear that the use of the sodium bicarbonate system to produce a soluble form of PC is most advantageous because of the good resolution obtained and ease of decarbonation to facilitate the isolation of sodium PC.

The availability of PC led to a study of its chemical lability. This study essentially agrees with earlier work by Meyer et al. (1959) but extends knowledge of the

lability of PC under certain conditions. Previous work has led to the grouping of phosphoric esters into three classes, depending on their lability, and include: (i) "free phosphate", compounds such as acetyl phosphate that hydrolyse very rapidly under acidic conditions, (ii) acid labile phosphate, compounds that hydrolyse after 15 min boiling in 1N H_2SO_4 such as nucleotide polyphosphates, and (iii) "residual" phosphate withstanding such treatment, including glucose 6-phosphate and adenosine 5-phosphate (Lindberg and Ernster, 1956). In this context PC is partially acid labile since it is <10% hydrolysed after 15 min at 100°C in 1N H_2SO_4 . In contrast, solutions kept at 0°C whether acidic or alkaline appear to be indefinitely stable.

The enzymatic lability of PC has also been studied and in this regard PC has been found to be cleaved by both acid and alkaline phosphatases. This important observation suggests that a coupled enzymatic assay for PC may be possible involving initial cleavage of PC into citrate and P_i by the action of phosphatases. Once cleaved citrate may be determined by published enzymatic assay techniques.

A further prediction to arise from the observation on the enzymatic lability of PC, is that in vivo PC may have a degree of lability.

2.6 SUMMARY

1. A synthesis and purification of PC has been described.

2. Of the phosphorylating reagents investigated, CEP appeared to be the most useful. o-Phenylene chlorophosphate was not suitable since oxidation gave rise to products

which hindered chromatographic techniques.

3. AG 1-X8 (HCO_3^-) columns were superior for the isolation of PC because of resolving power and ease of decarboxonation of buffers.

4. ^1H , ^{13}C and ^{31}P NMR provided structural proof of PC and further indicated that the synthetic method gives high purity PC.

5. PC hydrolysed in 3h at 100°C but was stable indefinitely at 0°C over a wide range in pH.

6. PC was labile to acid and alkaline phosphatases.

CHAPTER 3

FURTHER DEVELOPMENT OF SEPARATION AND ANALYTICAL SYSTEMS FOR PHOSPHOCITRATE

3.1 INTRODUCTION

In the preceding chapter, a chemical synthesis of phosphocitrate (PC) was described and the product characterised by physical and other methods. With the availability of PC as a reference material it was now possible to explore the development of systems for the potential purification and analysis of PC from natural sources.

Although sensitive tests exist for detecting and determining P_i (Brunette et al., 1978; Anner and Moosmayer, 1975; Penney, 1976; Lindberg and Ernster, 1956; Lanzetta et al., 1979) and indeed have proven satisfactory for analysing hydrolysed PC, selective PC analysis would be facilitated by the additional analysis of citrate. In fact, citrate is known to undergo a unique degradation, under well-defined conditions, to give pentabromoacetone (PBA). This reaction is the basis of selective assays resulting in varying degrees of sensitivity (Taylor, 1953; Ettinger et al., 1952); PBA gives a colour reaction with sodium sulphide (Taylor, 1923) or thiourea (Ettinger et al., 1952). A special modification developed by Jones (1967) has employed the Fujiwara reaction of halogenated hydrocarbons (Leibman and Hindman, 1964) to give a very sensitive chemical assay for citrate in the range 0-10 μ g. The major disadvantage of this method is the

requirement to use benzidine as a chromophore since it is a known carcinogen, and therefore undesirable for routine analysis. Consequently, attempts were made to find a suitable substitute for benzidine.

The strongly acidic conditions necessary for the chemical determination of citrate together with the temperature of reaction (ca. 50°C) indicated that PC would be hydrolysed during the assay. The chemical method then, is limited to the determination of total citrate since hydrolysis of PC leads to a background colour reaction. In contrast, Passonneau and Brown (1974) have developed a coupled enzyme assay system operating under mild conditions. With this technique citrate is cleaved to acetyl CoA and oxaloacetate (OAA) using citrate lyase (EC 4.1.3.6); then the OAA is reduced to malate by malate dehydrogenase (EC 1.1.1.37). The latter reaction involves the oxidation of NADH to NAD⁺ which can be monitored by fluorimetric (Passonneau and Brown, 1974; Lowry et al., 1961) or UV (O'Neill and Sakamoto, 1969; Costello and O'Neill, 1969) techniques. In the preceding chapter PC was shown to be cleaved by phosphatase activity; thus, providing PC is not degraded in any way by citrate lyase, then the possibility arises that alkaline phosphatase added to the standard coupled enzyme assay for citrate would afford a technique for analysing free citrate and PC. These aspects are reported in this chapter.

In biological samples it might be desirable to effect a preliminary separation of citrate from PC to enable PC determination. The ion-exchange system described in the preceding chapter was, in part, developed for this purpose

and also to enable the simple isolation of material from eluting buffers. Furthermore, since Howard (1976) has claimed that acidic conditions during column chromatography lead to artefact formation, the chromatographic method was developed to operate at a pH of 7. However, Howard did not directly isolate or quantitate PC to reach his conclusions. Consequently, this chapter describes a more rigorous investigation of the suggestion that acidification and neutralisation of a solution of citrate and phosphate can cause PC production. Clearly, in biological studies, where quantitation of PC is vital, it is important to know what level of artefactual PC production is present - if it occurs at all - and to elucidate the conditions responsible for such a reaction so that they may be avoided if at all possible.

Although chromatographic resolution of biological material on ion-exchange resins, together with chemical and enzymatic analysis of products is of value in identifying PC in nature, it is advantageous to have other techniques to confirm results obtained. In order, then, to facilitate comparison with synthetic PC, other methods were investigated including thin-layer chromatography (TLC), high-voltage electrophoresis, isotachophoresis, high-pressure liquid chromatography (HPLC) and gas-liquid partition chromatography (GLC).

Because of the wide range of techniques investigated and the extent of the data accumulated, some pertinent to the subsequent development of an analytical or separation technique, this chapter is organised so that individual techniques and their associated results are described

separately. Highlights and conclusions gained from these techniques are then incorporated into a general discussion.

3.2 METHODS AND RESULTS

3.2.1 ANALYTICAL TECHNIQUES

3.2.1.1 Citrate and PC

3.2.1.1.1 Chemical

Citrate was determined by conversion to the pentabromoacetone (PBA) derivative and followed by the Fujiwara reaction, as reported by Jones (1967). PBA was prepared as described earlier (Section 2.3.1.1) and 4ml aliquots of the organic phase were pipetted into tubes containing 2.5ml of a mixture of pyridine, water and tetramethyl ammonium hydroxide (60:20:0.8, v/v). The tubes were held in a water bath (ca. 50°C) while a stream of N₂ gas was directed into the tubes to evaporate the organic phase. The tubes were stoppered and then immersed in a boiling water bath for 3 min before cooling for 10 min in an ice bath. The colour reaction was initiated by the addition of 0.5ml of a solution of 1.5% recrystallised benzidine in 28% formic acid. The tubes were left standing for 1-3h at room temperature in the dark before measuring the absorbance at 530nm. Citrate standards (0-10 µg) were included routinely.

The method was not specific for free citrate, as PC itself was found to give a colour reaction (ca. 30%) in the PBA-citrate assay. Thus this method can measure reliably only total citrate. At 100°C in 0.1N H₂SO₄ PC is hydrolysed

completely after 4h. Accurately weighed samples of PC subjected to these hydrolysis conditions were found to contain the theoretical amount of citrate after chemical citrate analysis.

In order to find a replacement for benzidine, a range of aromatic amines was tested as chromophores in the citrate assay. After colour development UV spectra for each reagent were read against the appropriate blanks and the absorbance values determined over a range of wavelengths. The results are given in Figure 3.1. None of the reagents gave the intense colour observable with benzidine although aniline and 4,4'-methylenedianiline (MDA) gave strongly absorbing brown coloured solutions. Notably the commercial analogue of benzidine - 3,3',5,5'-tetramethylbenzidine - did not give a strong colour reaction. MDA was selected as a benzidine substitute since aniline requires distillation before use, whereas MDA can be used without recrystallisation.

A comparison of benzidine and MDA as chromophores is given in Figure 3.2. Benzidine causes the development of an intense colour reaction but above 10 μg citrate the colour is so intense that Beer's Law does not appear to be obeyed. MDA, in contrast, since it does not give such a strong colour reaction, gives a linear absorbancy with regard to the amount of citrate present over a larger range (0-25 μg).

These observations allow for the development of a flexible chemical citrate assay. Above a certain level of citrate, a pink colour develops in the pyridine phase during the assay. In this instance if benzidine was added the

Figure 3.1 Spectra of various chromophores after colour reaction in the citrate assay.

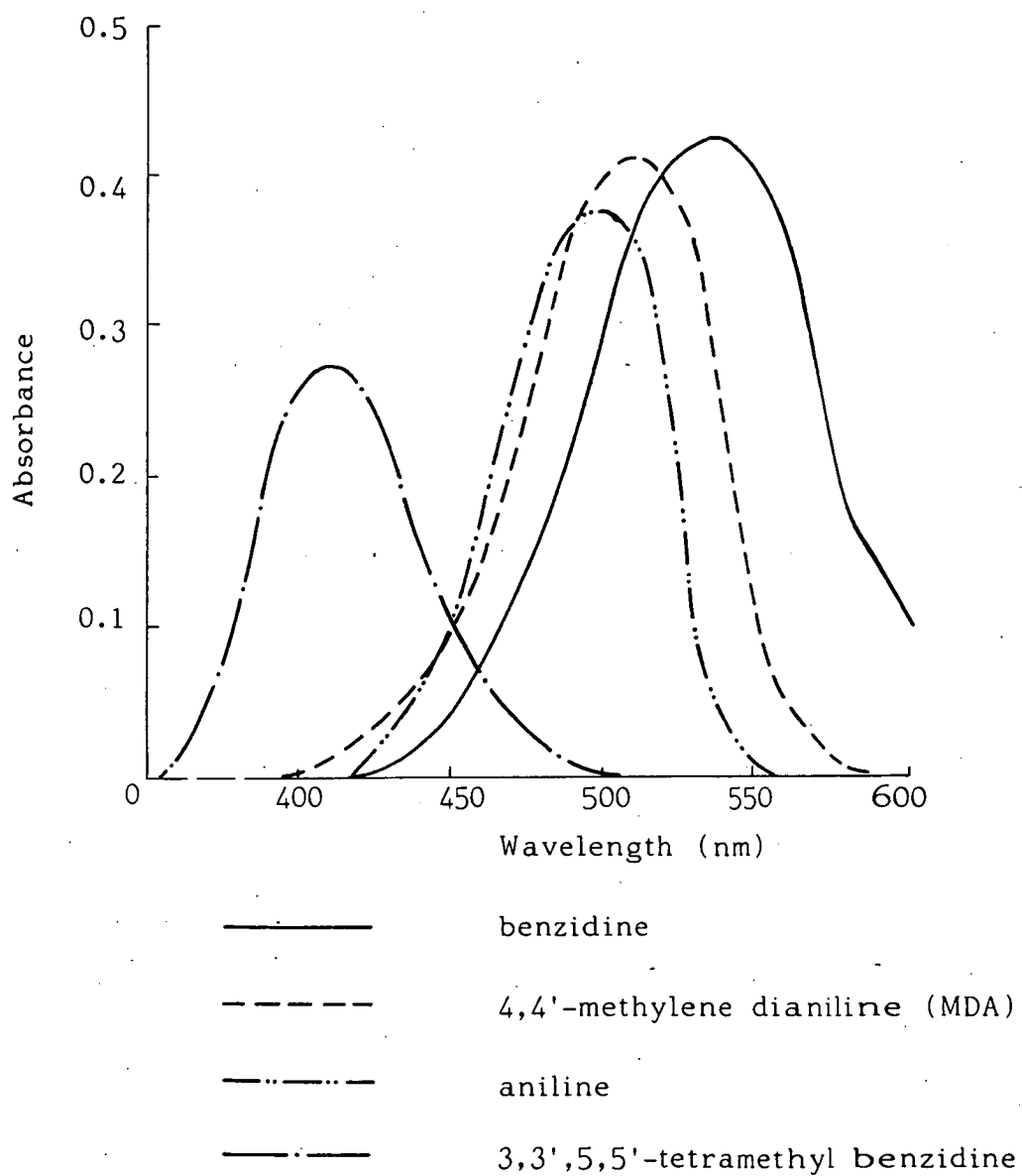
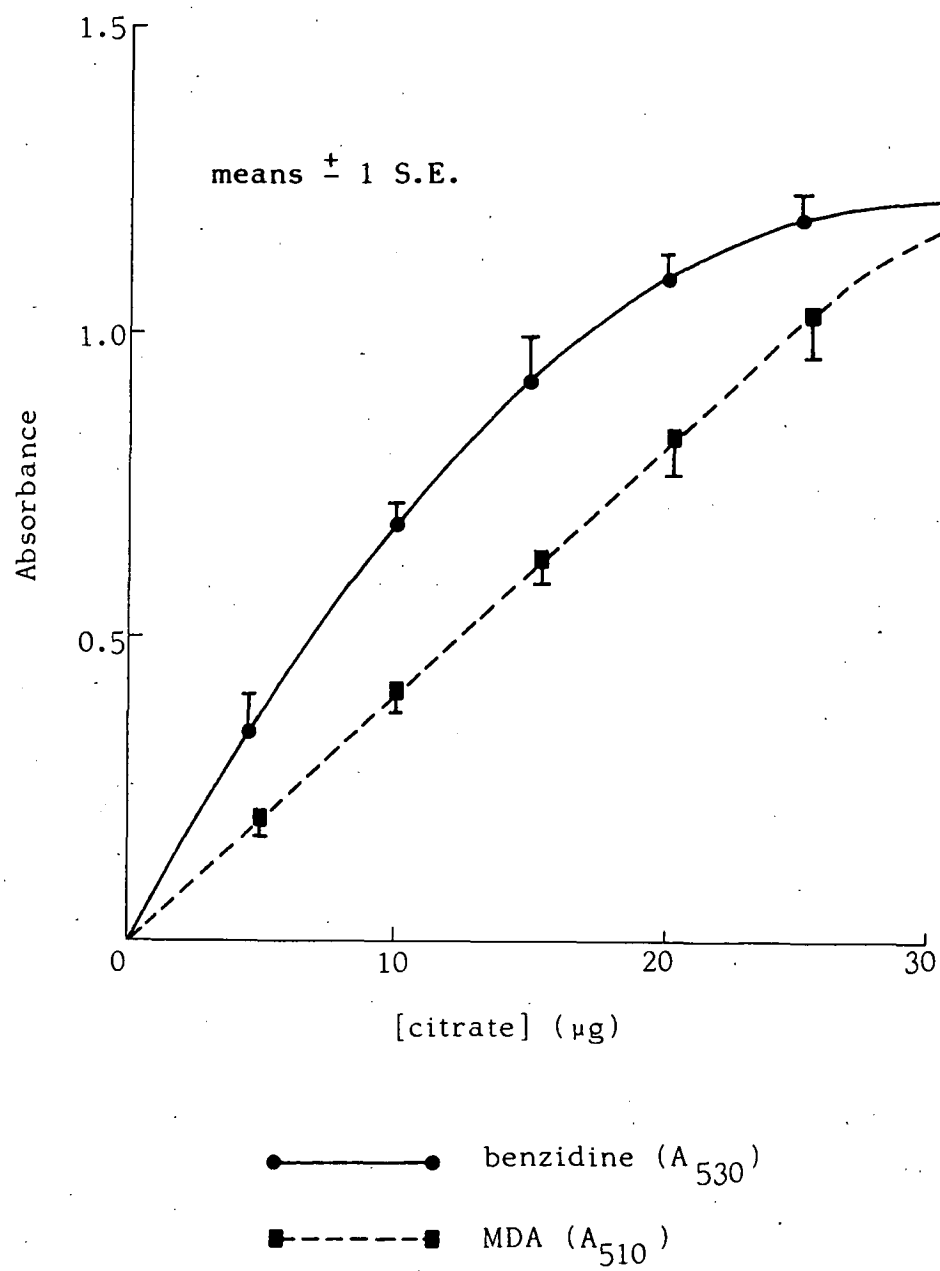


Figure 3.2 Comparison of benzidine and 4,4'-methylene dianiline as chromophores in the citrate assay.



absorbance would be too high for spectrophotometry. However, the addition of MDA would allow for accurate analysis. Thus the development of a pink colour in the pyridine phase acts as a convenient guide to the selection of the appropriate chromophore; benzidine is added only if no colour forms in the pyridine phase.

3.2.1.1.2 Enzymatic

Citrate was determined by measuring the NADH consumed using the coupled enzyme assay described by Passonneau and Brown(1974). PC was found not to be degraded by citrate lyase: PC did not cause an oxidation of NADH. Consequently, PC could be analysed after a pre-incubation with alkaline phosphatase since PC was cleaved into P_i and citrate. It was found that 10 μ l of alkaline phosphatase (calf intestine, Grade 1, 400U/0.17ml) added to ca. 50 μ g PC caused 100% hydrolysis after 15 minutes at 37°C and pH 10.4.

Accordingly, total citrate was determined by pipetting 1.0ml of 10mM glycine-KOH buffer (pH 10.4), up to 1ml of sample, and 10 μ l alkaline phosphatase into a 3ml cuvette (1 x 1cm light path) and incubating at 37°C for 10-20 min. After this period, 1.0ml of 100mM Tris-HCl buffer (pH 7.0), 10 μ l malate dehydrogenase (850U/ml), 0.2ml of NADH (8mg NADH in 10ml of 0.1M carbonate buffer, pH 10.6) were added. After thorough mixing, the absorbancy at 345nm was measured before the addition of 10 μ l citrate lyase reagent [2mg of the solid (2.2U/mg protein) in 1ml of 50mM triethanolamine buffer, pH 7.4, containing 0.03mM $ZnCl_2$ and 0.5M $(NH_4)_2SO_4$]. Within 10 min the absorbance decreased to a steady value.

Free citrate was measured by the omission of alkaline phosphatase from the mixture. The difference between total and free citrate gave an estimate of PC concentration.

By adjusting the amount of NADH added initially the method conveniently may be changed to a fluorimetric assay allowing 0-2 μg of citrate to be determined. Figure 3.3 gives the standard curves for citrate using the fluorimetric and UV techniques.

3.2.1.2 Phosphate and PC

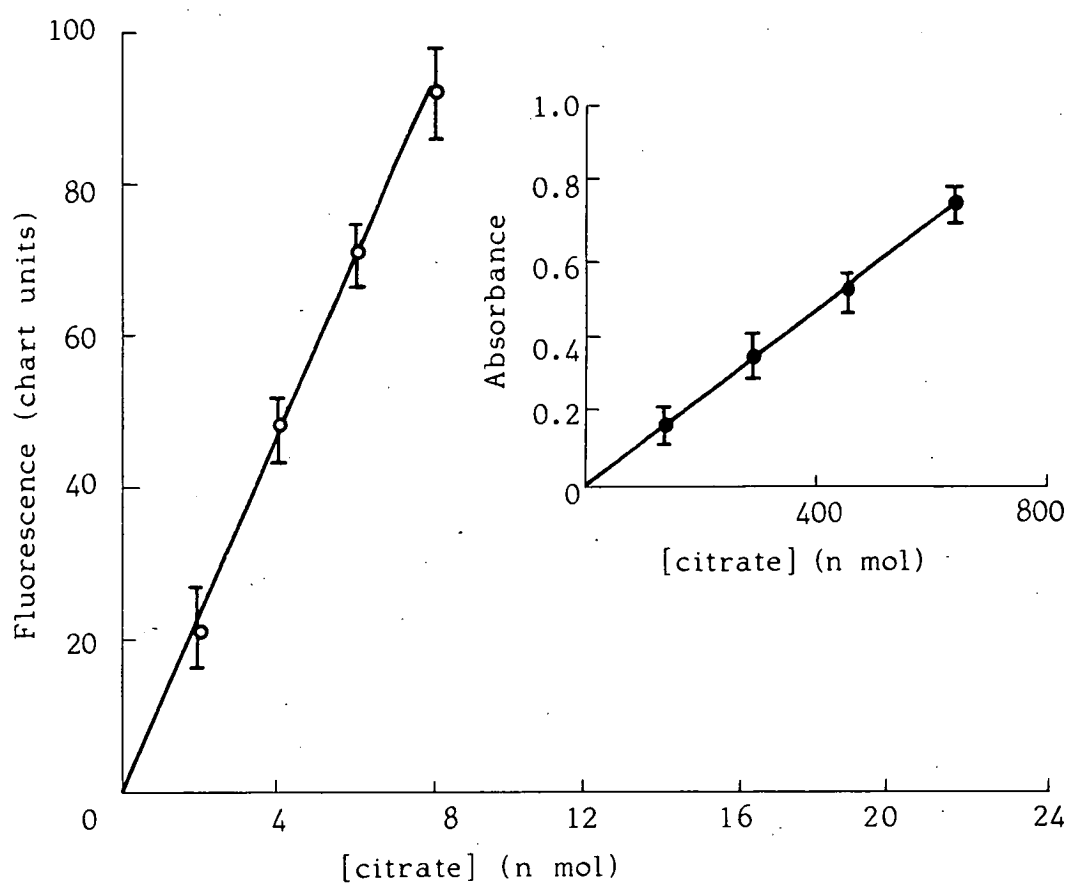
Phosphate was determined by the Fiske-SubbaRow method (Fiske and SubbaRow, 1925, 1929) as described in the preceding chapter (Section 2.3.1.2). Preliminary studies indicated that 4h in 0.1N H_2SO_4 at 100°C was sufficient to cause complete hydrolysis of PC to its components.

3.2.2 SEPARATION TECHNIQUES

3.2.2.1 Ion-exchange chromatography

Whereas batch elution was sufficient to resolve PC from contaminants in the synthetic procedure, greater resolution and accuracy appeared necessary for biological applications. Accordingly, gradient elution was investigated under precise conditions where material was loaded onto a 10 x 1.5cm resin column at a flow rate of 1ml min^{-1} at room temperature. PC (50 μg) and citrate (500 μg) were dissolved in water, the pH adjusted to between 7 and 8 and the solution loaded onto an AG 1-X8 (HCO_3^-) column. A batch elution with 600ml of 0.27M NaHCO_3 was made before a linear gradient was applied to the column. Five hundred ml each of 0.27M and 0.50M NaHCO_3 was used to provide the gradient. As shown in

Figure 3.3 Standard curves for citrate using either fluorimetric or UV detection



—●— UV : standard curve for [citrate]

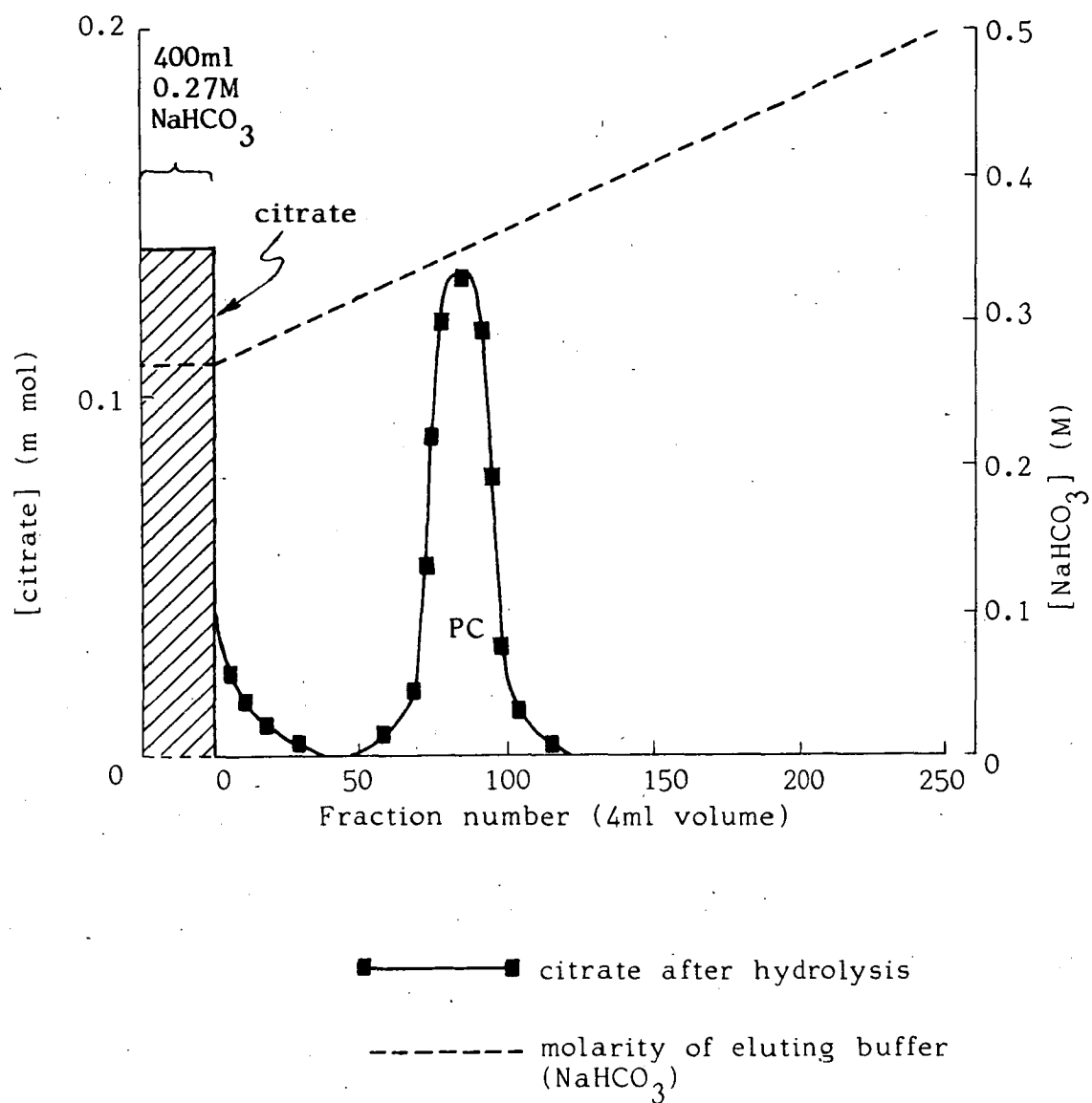
—○— fluorescence : standard curve for [citrate]

Figure 3.4, under these well-defined conditions, PC eluted characteristically after 300ml of gradient had been passed through the column. After the passage of a further 120ml, PC was completely removed from the column and there was no difficulty in reproducing this pattern. PC was detected by analysing either chemically or enzymatically for citrate released after hydrolysis.

In order to ascertain whether the acidification followed by P_i neutralisation of a solution of P_i and citrate led to PC production, 100mg each of citrate and P_i (as Na_2HPO_4) were dissolved in 20ml water and the pH adjusted to 1. After 15 min the pH was adjusted to 7, and the solution subjected to the exact chromatographic procedure described above. There was no evidence that PC or indeed any other citrate esters were formed. This result might be challenged on the basis that insufficient material was present for analysis, but when the tubes corresponding to the position PC eluted (i.e. 300-420ml) were pooled, decarbonated, and concentrated by freeze-drying, again there was no evidence that PC was present.

The use of triethylamine bicarbonate was investigated with a view to eliminating the decarbonation step involving the addition of AG 50W-X8 (H^+) since direct lyophilisation will remove the triethylamine salt. However, when small amounts of PC (50 μ g) were added to 50-100ml of triethylamine bicarbonate buffers, quantitative recovery was not obtained (<60%). This was in contrast to the full recovery demonstrable with sodium bicarbonate buffers. Presumably the amino group of triethylamine reacted with an acidic group on PC under

Figure 3.4 Typical elution profile of citrate and PC from an AG 1-X8 (HCO^-) column under standard operating conditions.



these dehydrating conditions to give a compound that did not react in the coupled enzyme citrate assay.

3.2.2.2 Thin-Layer Chromatography (TLC)

Glass plates were coated to a thickness of 0.25mm with cellulose (MN300HR from Machery-Nagel, Duren, Germany). After sample application, plates were developed in one of the experimental solvents before air-drying and staining with a relevant stain for citrate or P_i (Section 2.3.3).

Various solvent systems were tested for their ability to resolve P_i and PC. Some systems failed to give 'tight' spots, and of those that did (see Table 3.1) an isobutyric acid/5.5% NH_3 /MeOH solvent (60:40:100, v/v) gave the best separation of PC from P_i and citrate; the R_F 's were 0, 20, 0.42 and 0.44 respectively. Table 3.2 gives the R_F 's of a wide range of organic phosphates in this TLC system. Clearly if a phosphate stain is used, not all of the compounds can be resolved from one another. A citrate stain, however, will stain hydrolysed PC - pre-treated with an acid phosphatase solution - enabling PC to be distinguished from other phosphoric esters.

For routine analysis, mixtures were resolved using the isobutyric acid solvent and PC was detected by staining for citrate or P_i after hydrolysis with acid phosphatase.

3.2.2.3 High-voltage paper electrophoresis

High-voltage paper electrophoresis was performed essentially as described by Khwaja et al., (1970) as detailed earlier (Section 2.3.2). After electrophoresis, the paper was dried and spots visualised using the staining techniques

Table 3.1 R_F values obtained for the separation of P_i , citrate and PC by TLC using a range of solvent systems.

SOLVENT	R_F			COMMENTS
	CITRATE	P_i	PC	
EtOH	<u>ca.</u> 0.7	<u>ca.</u> 0.6	-	spots are streaked
EtOH/H ₂ O/formate (100:20:2 v/v)	0.85	0.61	-	spots are streaked
PrOH */H ₂ O/formate (100:20:2 v/v)	0.62	0.44	0	spots are streaked
EtOH/H ₂ O/triethylamine (100:20:2 v/v)	0.84	0	0	tight spot for citrate
Eucalyptol/PrOH*/formate/H ₂ O (50:50:20:10 v/v)	0.44	0.19	0.10	tight spots, but resolution of PC and P_i poor
Isobutyric acid/5.5% NH ₃ /MeOH (60:40:100 v/v)	0.42	0.44	0.20	tight spots and resolution of PC from P_i and citrate good

* PrOH = propanol

Table 3.2 R_F 's of phosphoric esters using an isobutyric acid/5.5% NH_3 /MeOH solvent system (60:40:100 v/v)

COMPOUND	R_F
P_i	0.44
ribose 5-P	0.34
AMP	0.32
ADPG	0.28
glucose 1-P	0.26
fructose 1, 6- P_2	0.26
glucose 1, 6- P_2	0.23
PC	0.20
ADP	0.13
ATP	0.05

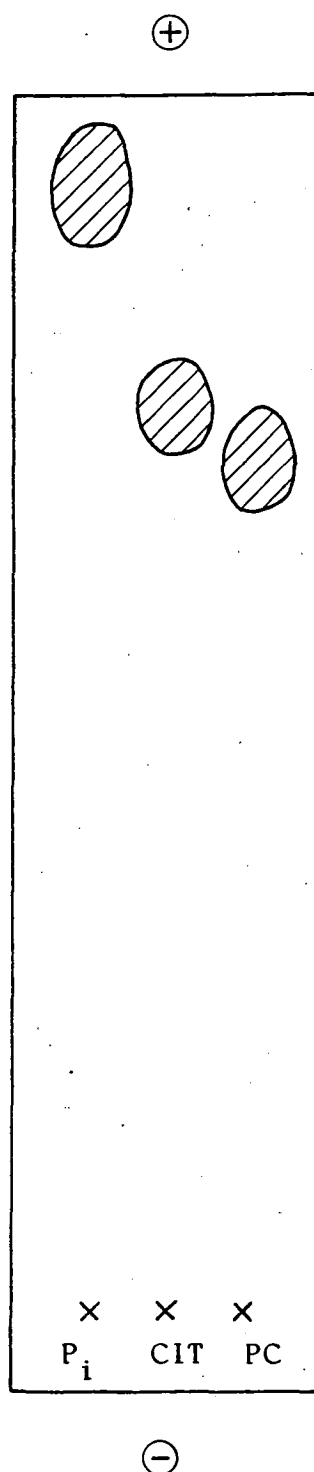
outlined before (Section 2.3.3). Figure 3.5 illustrates the electrophoretic resolution of P_i , citrate and PC. Although separation of PC from citrate is poor in this system, there is an absolute resolution of PC and P_i indicating that this method has particular application to the separation of [^{32}P]-labelled compounds. This conclusion was confirmed by electrophoresis of a range of phosphoric esters; PC was resolved from compounds which included nucleotides, fructose 1,6-diphosphate and phosphoglycerate. Relative mobilities are given in Table 3.3

3.2.2.4 Analytical isotachophoresis

Analytical isotachophoresis is a relatively new technique which will separate, for example, mixtures of inorganic anions (Bocek et al., 1977, 1978a), and metabolites such as Krebs cycle intermediates (Bocek et al., 1976, 1978b; Sjodin et al., 1975) without the need to form derivatives before separation. In capillary-type analytical isotachophoresis, samples are introduced between two electrolytes, termed leading and trailing buffers, for subsequent electrophoresis in the capillary tube. The sample ions are arranged in order of their mobilities into discrete zones and can be detected by UV, thermal or conductimetric techniques (Arlinger and Lundin, 1975; Brown and Hinckley, 1974; Delmotte, 1979; Everaerts et al., 1975, 1976).

The instrument used for isotachophoresis was made in these laboratories and is described schematically in Figure 3.6. This machine had facilities for both UV and conductimetric analysis as shown. A range of buffers was investigated and all buffers were

Figure 3.5 High-voltage paper electrophoresis of P_i , citrate and PC.



x origin

P_i = inorganic phosphate

CIT = citrate

PC = phosphocitrate

Table 3.3 Mobilities of phosphoric esters relative to P_i under conditions of high-voltage electrophoresis

COMPOUND	RELATIVE MOBILITY
P_i	1
3-phosphoglycerate	1
fructose, 1,6- P_2	0.90
PC	0.72
glucose 6-P	0.65
pyrophosphate	0.55
AMP	0.45
ADP	0.45
ATP	0.43

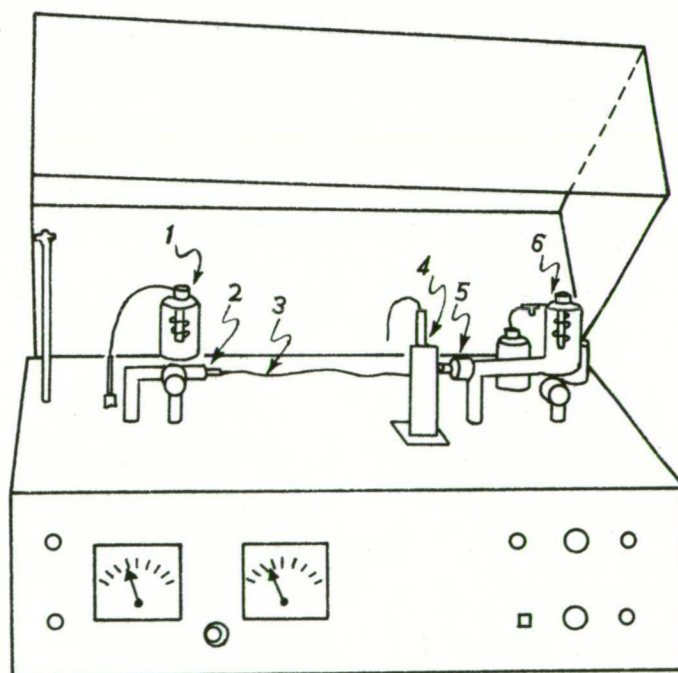
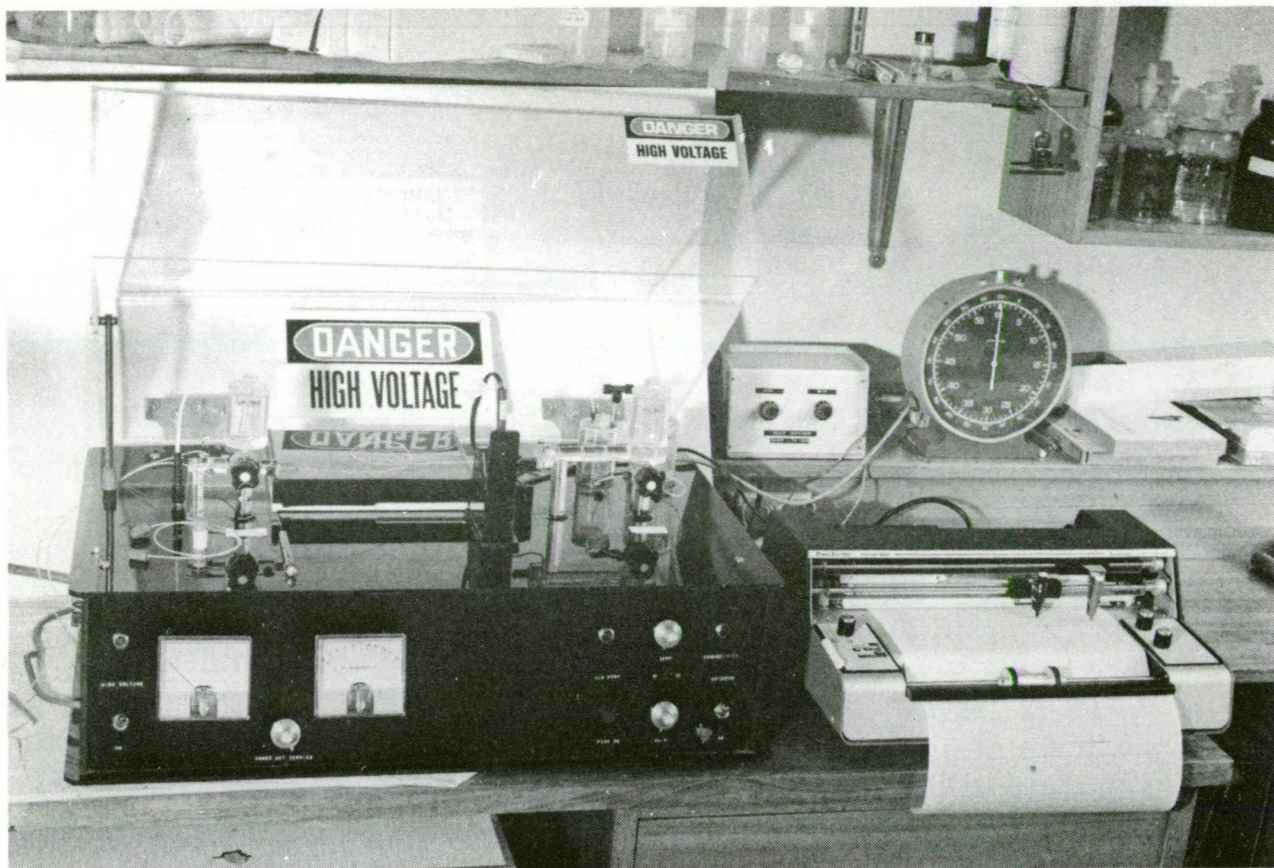


Figure 3.6 Schematic diagram of instrument used for analytical isotachopheresis.

- 1 = trailing buffer
- 2 = injection port
- 3 = capillary tube
- 4 = UV detector
- 5 = conductimetric electrode
- 6 = leading buffer

thoroughly degassed before use. Samples were pre-treated with AG 50W-X8 (H^+) to remove divalent cations which appeared to cause solubility problems. After freeze-drying the sample was dissolved in a minimum amount of liquid and 2-30 μ l injected. The migration current was set initially at 200mA and reduced to 50mA for the major period of the run and for recording purposes. The running time was between 25 and 50 min, depending on the length and internal diameter of the capillary tube.

Table 3.4 summarises results on the resolution of P_i , citrate and PC over a wide pH range. The systems providing the best resolution of these components operated at a pH of 4.7. Two buffer systems could be used to give this pH. One used 10mM HCl/aniline [0.2% hydroxypropylmethyl cellulose (HPMC)] as the leading electrolyte and 5mM glutamic acid (0.2% HPMC) as the terminator. An alternative system used 6-aminocaproic acid (0.2% HPMC) as the leading electrolyte, and 5mM pivalic acid as the terminator. With this latter system, PC, citrate, P_i and pivalic acid migrate in that order and give characteristic step heights of 25, 40, 50 and 75mm respectively, as shown in Figure 3.7. If aniline is used, oxidation products are formed and the traces are often unstable.

Analytical isotachophoresis will resolve and quantitate microgram amounts of PC in mixtures, but the technique does not completely separate samples with a high salt concentration. This disadvantage can be partially alleviated by using AG 1-X8 (HCO_3^-) ion-exchange column chromatography, and subsequent decarbonation with AG 50W-X8 (H^+) to give

Table 3.4 Resolution of PC, P_i and citrate by analytical isotachophoresis at different pHs.

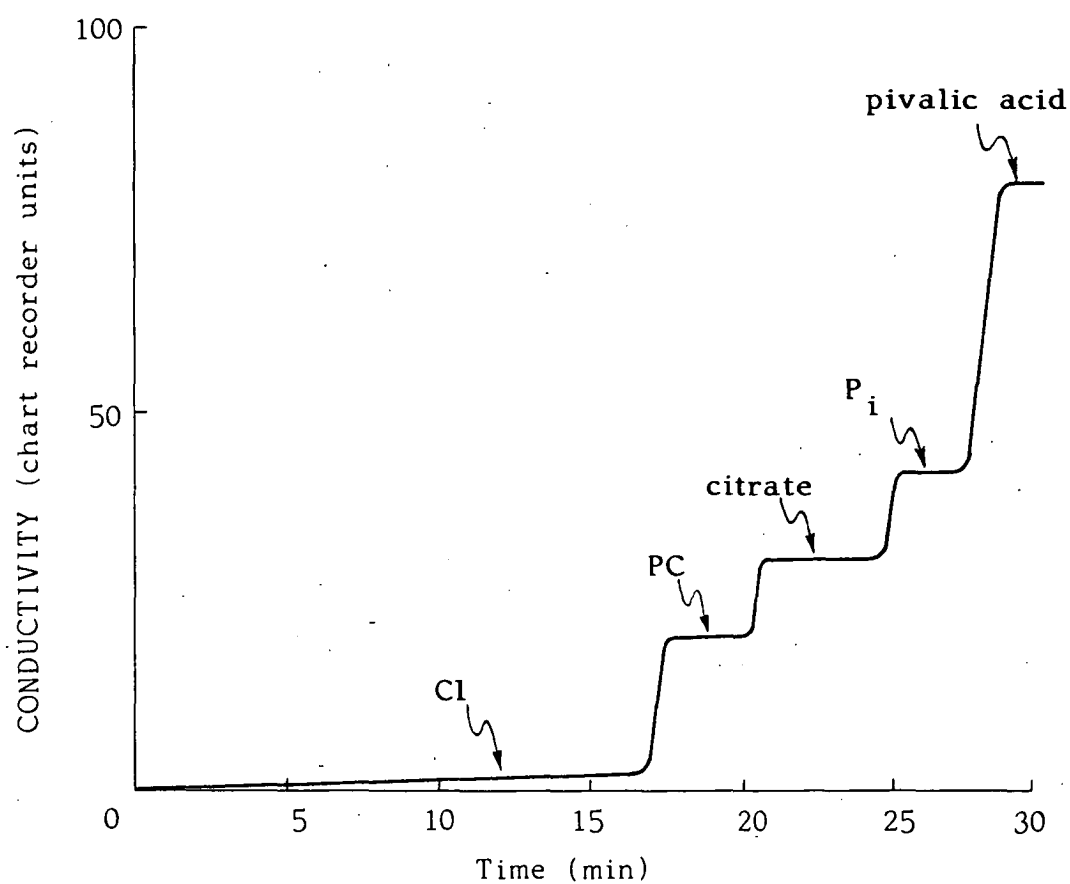
	STEP HEIGHT (mm)	TIME ⁺ (min)	STEP HEIGHT (mm)	TIME ⁺ (min)	STEP HEIGHT (mm)	TIME ⁺ (min)	STEP HEIGHT (mm)	TIME (min)	STEP HEIGHT (mm)	TIME (min)	STEP HEIGHT (mm)	TIME (min)
PC	22	18	26	24	30	25	25	17	24	20	22 [‡]	26 [‡]
P _i	27	31	40	44	45	40	40	21	36	30	47	72 [‡]
Citrate	36	55	52	94	60	52	50	25	54	52	22 [‡]	26 [‡]
Terminator	84	68	73	109	95	70	75	30	88	63	65	80
Leading* terminating	glycine HCl acetate		β-alanine HCl acetate		aniline HCl glutamate		6-aminocaproic acid HCl pivalic acid		β-alanine HCl glutamate		histidine HCl glutamate	
pH	3.5		4.5		4.7		4.7		4.8		6.0	

* leading = leading electrolyte (10mM), terminating = trailing electrolyte (5mM), all solutions with 0.2% hydroxypropyl methyl cellulose.

⁺ times vary with salt concentration, concentrations of migrating ions and length of capillary tube.

[‡] co-migration of PC and citrate.

Figure 3.7 Isotachophoretic separation of P_i , citrate and PC.



desalted, cation-free samples.

3.2.2.5 High Pressure Liquid Chromatography (HPLC)

High pressure or high performance liquid chromatography (HPLC) involves passage of substances to be resolved through a microparticulate separating column at pressures normally greater than atmospheric, enabling efficient resolution to be obtained (Scott, 1978; Tomlinson *et al.*, 1978; Santi *et al.*, 1975; Eksborg *et al.*, 1973; Hearn and Hancock, 1979). Components once separated are commonly detected by UV, fluorimetric or refractometric techniques, the first two being the most sensitive. Unfortunately in the present application - the separation of P_i , citrate and PC - UV and fluorimetric detection systems are of little use since these ions contain no UV-absorbing or fluorescent moieties. Although, at 210nm carbonyl moieties absorb to a small degree, 10 μ g of citrate, for example, is the threshold for detection (Palmer and List, 1973; Turkelson and Richards, 1978). One method to increase sensitivity is to tag anions with a UV-absorbing counter-ion as in the ion-pairing straight phase chromatographic system developed by Lagerstrom (Lagerstrom, 1975; Eksborg *et al.*, 1973). However, this particular method has not been extended to such hydrophilic molecules as the polyacids citrate, P_i and PC. Indeed P_i is used as an inert ion in the system indicating the difficulty that would be involved in applying this method to the present requirements.

Another method facilitating UV-labelling is to attach phenacyl moieties to acidic groups using the crown ether

method. Not only does this method permit UV detection, but it has also enabled derivatised samples to be resolved by reversed phase HPLC. This method has been applied to dicarboxylic acids (Grushka et al., 1975), but not to tricarboxylic acids. Whether phosphate or phosphoric acid moieties would be derivatised is as yet unknown. As a final consideration, the extended periods of heating required for derivatisation suggests it cannot be applied to PC analysis because of the lability of PC at higher temperatures.

From experiences with the conventional ion-exchange systems, preliminary investigation centred on the possibility of using an anionic column to enable the HPLC separation of P_i , citrate and PC. Furthermore, since NO_3^- had given the best resolution with conventional techniques, this was used as the eluting ion with HPLC. Waters Associates equipment was used consisting of two Model 6000A Chromatographic Solvent pumps, a Model 440 Absorbance Detector, a Differential Refractometer Electronics Unit and a Model 660 Solvent Programmer. Samples (10-50 μ l) were loaded onto an anionic AX column before gradient elution. All buffers were filtered and degassed before use. Using a programmed sequence of flow rates, together with a gradient pump facility (linear from 0 to 0.2M $NaNO_3$), an attempt was made to resolve anions. Unfortunately components could not be detected by UV due to the high background from NO_3^- buffer; the minimum sensitivity of diffractometry was $> 50 \mu$ g citrate. In fact, the use of gradients rendered diffractometry difficult so that fractions had to be collected, hydrolysed, and then analysed

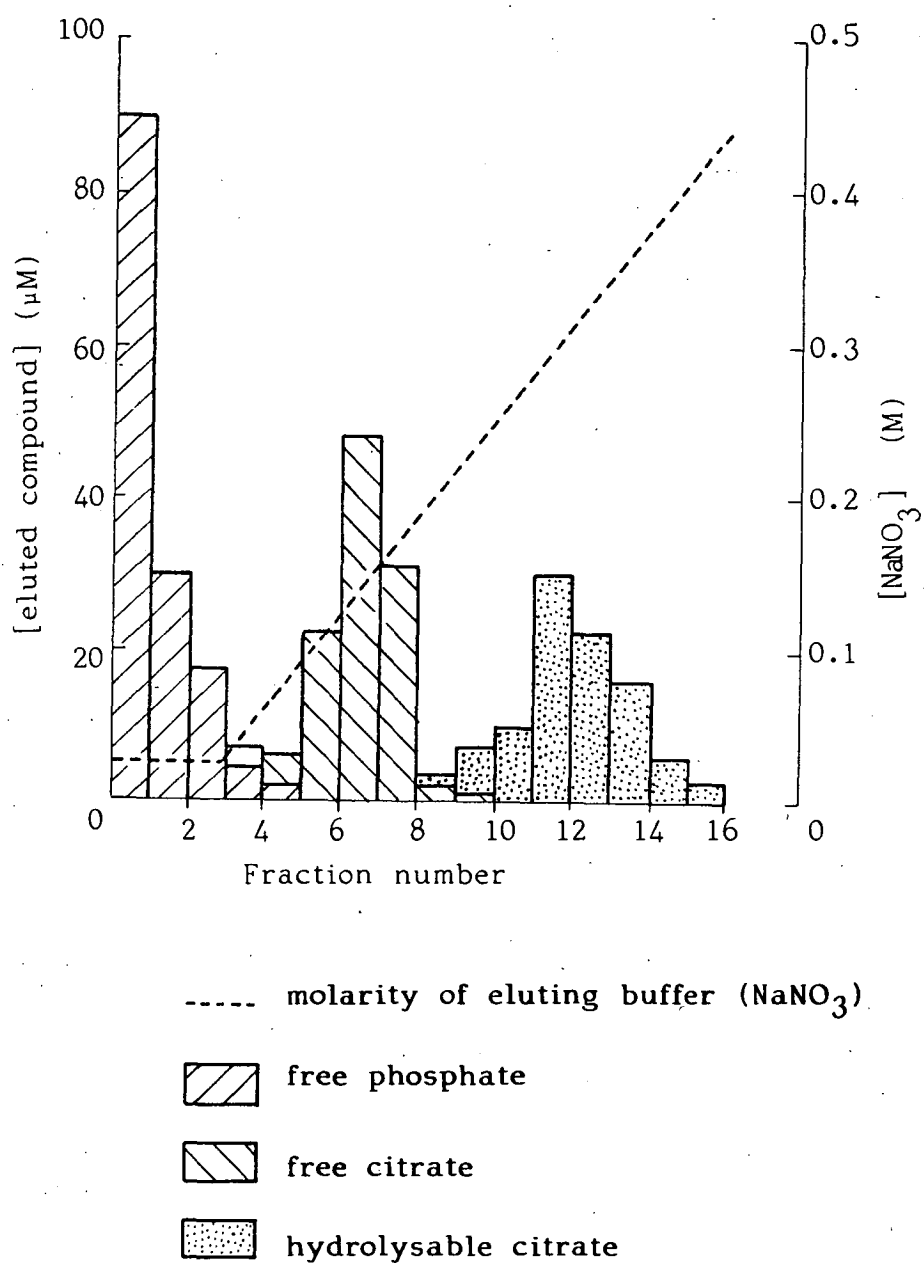
for phosphate and citrate. The result from such an experiment is shown in Figure 3.8 indicating that HPLC will resolve the mixture of anions - P_i , PC and citrate. However, the difficulty of detection makes this method of little advantage over conventional ion-exchange and adds the disadvantage that only small amounts can be resolved at any one time.

3.2.2.6 Gas-Liquid Partition Chromatography (GLC)

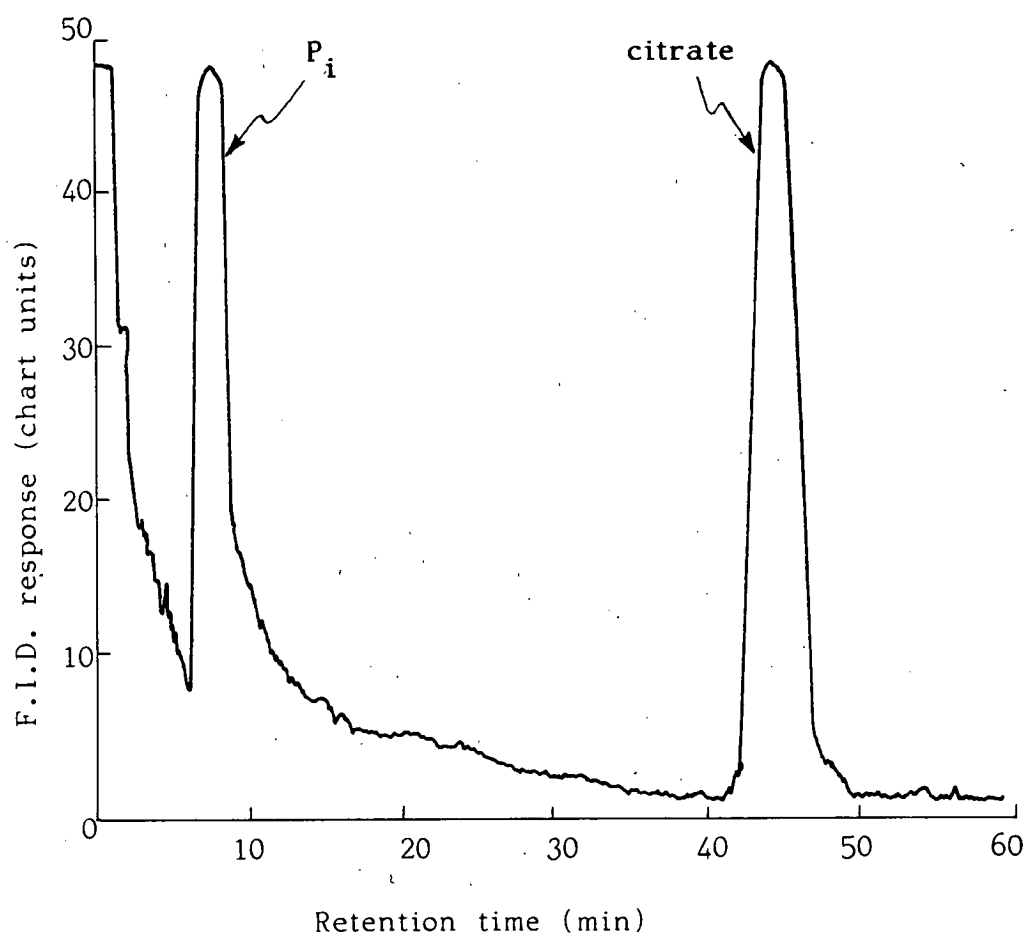
GLC requires the derivatisation of non-volatile components so that separation can be effected. Quantitative trimethylsilylation of citrate has been described by Dalglish *et al.* (1966). Accordingly, 1mg of cation-free sample was dissolved in a minimum of pyridine (50-100 μ l) and 200 μ l of a mixture of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMS) (from Pierce Chemical Co., Rockford, U.S.A.) added. After 30 min 2-10 μ l aliquots were injected into a Varian Aerograph 1520 machine fitted with 1 12% DC-QF 1 on Gas Chrom Q column and an F.I.D. detector. The injector temperature was 180°C, column temperature 180°C and the detector temperature 200°C. The gas flow (N_2/H_2) was set at ca. 10ml min⁻¹ before calibration. Under these conditions the retention times of pyrophosphate, P_i , citrate and TEC were found to be 2, 8-9, 40-48 and 50-60 min respectively.

When PC, treated with HMDS/TMS/pyridine mixture, was injected into the column, only citrate and P_i were detected (Figure 3.9). When pyridine was omitted, no peaks were observed with PC. A reason for this apparent decomposition of PC might be the use of the free acid of PC rather

Figure 3.8 Separation of P_i , citrate and PC by HPLC using an anionic (AX) column.



Figurer 3.9 Evidence for the lack of stability of PC after derivatisation for gas chromatography.



than the more stable sodium salt.

In an attempt to avoid these problems, various other methods for trimethylsilylation were investigated. A milder method developed specifically for the derivatisation of phosphoric esters (Hashizume and Sasaki, 1966) requires dissolving the sample in dimethylsulphoxide (DMSO), addition of cyclohexane to extract the derivatised sample, and addition of the trimethylsilylating reagent. When PC was subjected to this procedure, a yellow colour appeared in the DMSO phase. This was not observed when other test phosphoric esters were derivatised; whereas these compounds gave single peaks after GLC, no PC peak was detectable. No PC peak was detectable using other reagents (Pierce Chemical Co., Rockford, U.S.A.) including N-trimethylsilyldiethylamine, N-trimethylsilylimidazole and N,O-bis(trimethylsilyl)acetamide.

These data suggest that PC is unstable to trimethylsilylation. Consequently, other applicable derivatisation procedures were sought (Nicholson, 1978a, b; Cram, 1978). The most promising appeared to be methylation since this involved the introduction of a relatively small group into a sterically crowded array of acidic groups. Methylation can be effected by either a thionyl chloride/MeOH mixture or by using diazomethane, for example. The former will give complete esterification of polyacids only if heated at 60°C for 20-30 min, conditions that will hydrolyse PC. The reactivity and difficulty of handling diazomethane led to the avoidance of this method, but the more recent work by Tew *et al.* (1980) indicates that complete derivatisation of PC was not possible under the conditions used.

3.3 DISCUSSION

A number of techniques, both analytical and preparative, have been investigated for purifying and quantitating PC exploiting various characteristics of the PC molecule. The data presented here indicate that the AG 1-X8 (HCO_3^-) ion-exchange chromatographic system developed affords definitive separation of PC from citrate and that PC elutes at a characteristic volume upon linear gradient elution. Quantitative recovery is possible using sodium as the cation but with triethylamine this is not possible, presumably because coupling with PC occurs under the dehydrating conditions of lyophilisation, rendering citrate undetectable using enzymic assay.

The development of this system enabled a rigorous examination of the claim by Howard (1976) that acidification and neutralisation of a sample of P_i and citrate leads to PC production. In his study the compound was not isolated or characterised. Rather, the indirect evidence of a rise in the anti-calcifying potential of the mixture was taken as an indication of the production of a new calcification inhibitor. The increase in molar strength due to acidification and neutralisation is known not to significantly affect inhibition (Termine and Posner, 1970; Termine *et al.*, 1970); thus the new compound was proposed to be PC.

Sutor *et al.*, (1979a), however, have questioned whether PC can be formed in this way, claiming that chemical arguments cannot substantiate such an artefactual formation. This claim, however, is not valid as known organic reactions

would in fact support such a method of PC production (Figure 3.10). For example, the S_N1 and $E1$ reactions of t-butyl alcohol in aqueous sulphuric acid give t-butyl sulphate and t-butylene respectively. An S_N1 reaction of P_i and citrate can thus be foreseen to give PC by a closely analogous process. Furthermore, S_N1 reactions are favoured by solvents with a high dielectric constant, for example, water, allowing stabilisation of ions formed; and tertiary alcohols inductively stabilise intermediate carbanions (Roberts and Caserio, 1965). Consequently the reaction of citrate, a tertiary alcohol, in dilute aqueous phosphoric acid is optimally suited to produce PC contrary to the theoretical predictions of Sutor *et al.* (1979a). As a further indication of the likelihood of the artefactual formation of PC, the $E1$ reaction of citrate to give aconitate in acid is well known (Howard, 1976; Roberts and Caserio, 1965).

Nevertheless, in the present study no evidence could be found supporting the idea that acidification and neutralisation of P_i and citrate leads to PC production. Instead of relying on indirect evidence, samples from ion-exchange chromatographic columns were pooled, decarbonated, and freeze-dried before direct enzymatic and chemical analysis for PC. The amounts of P_i and citrate used in these experiments were in excess over the levels calculated to be present in biological samples of less than 100g, indicating that tissue samples can be subjected to TCA extraction followed by KOH treatment without spurious PC production. The question of the inclusion of PC into the KIO_4 deposit remains unanswered

at this stage, but could be readily investigated using [^{32}P]-PC (see Section 6.3.1).

PC, once separated, can be determined by either the chemical or enzymatic techniques developed here. An alternative chromophore to the commonly used benzidine is suggested to be 4,4'-methylene dianiline (MDA) which is less toxic, and although not giving the same intense colour reaction as benzidine, it does allow estimation of a wider range of citrate concentrations. These observations have allowed a versatile citrate assay to be developed: benzidine is used to detect low citrate concentrations (0-8 μg) and MDA to detect a wider range of citrate levels (0-20 μg).

A limitation of the chemical citrate assay is the partial reaction that occurs with PC itself. Consequently, only total citrate can be measured accurately by this method so that complete separation of citrate and PC by column chromatography is obligatory. As an alternative the coupled enzyme system described here is more flexible. PC is not cleaved by citrate lyase but since it is labile to both alkaline and acid phosphatase, measurement of free citrate and PC is possible. Microgram and submicrogram quantities can be determined using fluorimetric techniques, provided background fluorescence is minimal.

A list of the techniques useful for characterising PC is given in Table 3.5. HPLC and GLC are not listed because of difficulties involved in the application of these techniques. The trimethylsilylating methods necessary for GLC appear to lead to the decomposition of PC. Furthermore, even milder techniques such as methylation are not effective in fully-

Table 3.5 Techniques for identifying phosphocitrate.

SYSTEM	DETAILS	COMMENTS
Ion-exchange chromatography	AG 1-X8 (HCO_3^-), pH 7.2-7.4	batch or gradient elution with definitive separation of P_i , citrate and PC
Thin layer chromatography	Cellulose: isobutyric/5.5% NH_3 /MeOH (60:40:100 v/v)	R_F values: PC (0.2), P_i (0.44), citrate (0.42)
High-voltage paper electrophoresis	3MM: 0.05M triethanolamine- HCO_3^- , pH 7.5, 2000v/2hr	distance from origin (cm): PC (23), P_i (32), citrate (25)
Enzymatic	coupled assay with phosphatase, citrate lyase and malate dehydrogenase - UV or fluorimetric	PC sensitivity: 0.2-100 μg
Chemical	PBA derivative with benzidine	PC sensitivity: 0.5-5 μg
Isotachophoresis	aminocaproic acid/pivalic acid system	step height (mm): PC (25), citrate (40), P_i (50), pivalic acid (75); PC sensitivity: nmoles

derivatising PC (Tew et al., 1980). With HPLC, present detection techniques make unreacted PC difficult to detect. The HPLC anionic column separation developed here is therefore limited, requiring the collection of fractions and subsequent analysis of tube contents to identify separated components. The use of ion-pair reversed phase HPLC could not be used to improve detection since if a UV absorbing counter ion was used the background UV absorption would be too high for the detection of resolved components. The only techniques that could, in theory at least, improve sensitivity are phenacyl derivatisation of PC, or straight-phase ion-paired chromatography. The former, however, requires derivatisation conditions that would hydrolyse PC and the latter technique is presently only of use with relatively hydrophobic molecules, certainly not with such hydrophilic entities as PC, P_i , and citrate.

One technique that does not require UV absorption for sensitivity in detection, and in fact works optimally for highly charged anions, is isotachophoresis. As an analytical technique this method has great applicability since nanomole amounts of PC are detectable and PC, furthermore, is characterised by a unique step height reflecting the particular conductivity of the migrating anionic zone. The width of the zone is proportional to the amount of material present enabling quantitation.

Isotachophoresis is ideal for pure solutions; but technology has not yet been perfected to quantitate compounds in the presence of high salt concentrations. Thus the caproic acid/pivalic acid system described here offers the possibility

of at least confirming the presence of PC in biological samples, if indeed it is present.

Another useful technique is TLC which, by using specific phosphate and citrate stains, will separate PC from citrate and P_i , and allow for its visualisation after cleavage with acid phosphatase. The limitation of TLC is that only small volumes of samples can be applied, a limitation which is not quite as restrictive with high-voltage paper electrophoresis. This latter technique permits good separation of PC and P_i using a triethylamine-bicarbonate buffer, although separation between citrate and PC is not definitive. However, alteration of running conditions or even second dimension electrophoresis may resolve PC and citrate; but at present, staining for phosphate will selectively stain PC and not citrate. Electrophoresis separates PC from a variety of other P_i -esters and these data suggest that this method is probably best suited to experiments where it is necessary to monitor [^{32}P]-labelled components.

If a compound gives a positive reaction for PC in more than one system then its identity as PC is more assured than a positive reaction in just one system, since each system has some limitations. Consequently, the techniques developed here allow not only the separation and determination of the citrate content of a "PC fraction", but, by comparison with authentic PC, the more confident assertion that the compound is indeed PC.

3.4 SUMMARY

1. Total citrate in PC can be determined by using the chemical PBA-citrate assay; however, PC was found to

be hydrolysed under the conditions, so free citrate cannot be accurately measured.

2. 4,4'-methylenedianiline was found to be a substitute for benzidine and the use of both reagents has allowed the development of a flexible citrate assay.

3. Free citrate and PC can be measured by a coupled enzyme system using alkaline phosphatase to cleave PC. Submicrogram amounts of PC can be determined.

4. An ion-exchange chromatographic system was developed that eluted PC from a column at a characteristic volume.

5. Acidification and neutralisation of citrate and P_i was not found to lead to PC production.

6. A TLC system was found that separated PC from P_i and citrate; the R_F 's were 0.20, 0.42 and 0.44 respectively.

7. High-voltage paper electrophoresis at pH 7.4 was demonstrated to resolve a wide range of phosphoric esters. Separation of PC and citrate was poor.

8. A variety of systems for analytical isotachopheresis were investigated. Using an aminocaproic acid/pivalic acid system, best separation and detection of PC, P_i and citrate was obtained.

9. HPLC with an anionic ion-exchange column (NO_3^-) separated PC, P_i and citrate but detection thresholds were too high for this technique to be of great use.

10. Under a wide range of derivatisation conditions, GLC could not detect PC.

CHAPTER 4

ANTI-CALCIFYING POTENTIAL OF PHOSPHOCITRATE

4.1 INTRODUCTION

The availability of highly-purified PC enables investigation of its ability to inhibit both calcium phosphate and calcium oxalate precipitation. These experiments are particularly important since Lehninger (1977) has cautioned that the powerful activity shown by biological fractions might not be due to PC but to some other factor(s) present. Further, whereas Howard (1976) has claimed "factor X" - presumably PC - inhibits HAP formation, Tew and Mahle (1977) have reported that the "purified factor" only inhibits if citrate is present. Work in this chapter is aimed at clarifying these conflicting reports and at elucidating the inhibitory characteristics of PC which might indicate whether PC should be considered as a new biological factor influencing biological calcification systems.

Calcium phosphate deposition can occur in association with the formation of renal stones, mitochondrial granules and the hydroxyapatite (HAP) of bone (Posner, 1973; Bygrave, 1978; Fleisch, 1978). Amorphous calcium phosphate (ACP) is recognised as the precursor of HAP both in vitro (Meyer and Eanes, 1978a, b) and in vivo (Dougherty, 1978; Posner and Betts, 1975). Test systems have been established based on the fact that when ACP hydrolyses after a specific time to the more basic phase (HAP), the acidity that develops in solution can be neutralised by controlled base titrimetry.

This type of system has been used to determine whether particular compounds can inhibit HAP formation and as a result compounds such as ATP, pyrophosphate, phosphitin and citrate have been established as inhibitors. The structural variety of compounds known to inhibit has led to little progress in understanding how the structure of a molecule affects its ability to inhibit.

Limited studies by Robertson and Fleisch (1970) have led to the suggestion that compounds possessing an $H_2O_3P-X-PO_3H_2$ structure (X may be C, N or O) will inhibit. The findings that fructose 1,6-bisphosphate and glycerol 2,3-bisphosphate also inhibited has caused Termine and Conn (1976) to broaden this initial concept proposing that "if inhibitor P-O-P bonds are not present, low molecular weight metabolites containing two ester phosphates (but not monophosphate or carboxylic acid groups) also inhibit". Clearly PC does not comply with this structural criterion. If then PC does show inhibitory properties then a re-appraisal of the structural factors influencing inhibitory ability is warranted.

Another important calcium salt in man is calcium oxalate. Whereas calcium phosphate is quantitatively more important, calcium oxalate is a major component of renal and urinary stones (King, 1971). If PC is to play a role in the prevention of lithiasis it should inhibit not only calcium phosphate deposition but also calcium oxalate crystallisation - a phenomenon as yet unstudied. The effect of PC on calcium oxalate precipitation was investigated using two experimental systems. One system involved the growth

of calcium oxalate on glass fibres; the other involved seeding a metastable solution of calcium oxalate. The use of two systems then has enabled a more extensive investigation of the inhibitory activity of PC.

Comparison of the inhibitory ability of PC with a range of well-known inhibitors was also undertaken. These inhibitors included ATP, EHDP, pyrophosphate, citrate and chondroitin sulphate C. These were tested in the calcium oxalate and/or calcium phosphate test systems, allowing the ability of PC to inhibit the deposition of either of the calcium salts to be compared.

The addition of a single compound to calcification systems allows any inhibitory ability to be revealed; but such an effect cannot be an accurate reflection of the way the compound acts in vivo, where many cellular and chemical interactions may influence inhibition. Synergism and other interactions have been investigated with calcium phosphate precipitation but there is no evidence from calcium oxalate studies. For example, Blumenthal et al. (1977) have shown that Mg^{++} has a synergistic effect with ATP on the inhibition of HAP formation. From preliminary work using impure PC, Howard (1976) has postulated that perhaps citrate and PC formed another synergistic couple. An attempt has been made to study these effects in both calcium phosphate and calcium oxalate systems, because of the important implications of such interactions to the physiological situation. In addition, the adsorption of PC onto HAP and calcium oxalate was also investigated, since inhibition is usually considered to involve adsorption onto active growth sites.

4.2 MATERIALS

Phosphocitrate was prepared as described earlier (Section 2.4.1.2) and imidodiphosphate (PNP) as described by Yount (1974). Ethane 1-hydroxy diphosphonate (EHDP) was a gift from Proctor and Gamble, Cincinnati, U.S.A. Adenosine phosphates were products of Calbiochem, Sydney, N.S.W. Hydroxyapatite was from Bio-Rad Laboratories, Richmond, California, U.S.A. Water was decarbonated and distilled before use. All other chemicals were reagent grade and obtained from commercial sources.

4.3 METHODS

4.3.1 CALCIUM PHOSPHATE PRECIPITATION

The transformation of ACP into HAP was followed titrimetrically essentially as described by Meyer and Eanes (1978a). Using a 1L flask, 0.5ml portions of 0.50M CaCl_2 and 0.50M NaH_2PO_4 were added to 600ml of stirred, high-purity decarbonated water and equilibrated in a water bath at 25°C. Sufficient 2M NaOH was then added over 60s to adjust the pH to 7.4. The mixture was maintained at this pH throughout the course of the reaction by using a pH-stat (Radiometer, Copenhagen, Denmark) and CO_2 was excluded from the system by bubbling N_2 .

4.3.2 CALCIUM OXALATE PRECIPITATION

4.3.2.1 Glass fibre system

Calcium oxalate crystals were grown on glass fibres essentially as described by Sutor (1969) and modified by Sallis and Lumley (1979). Glass threads (20 x 0.2mm) were

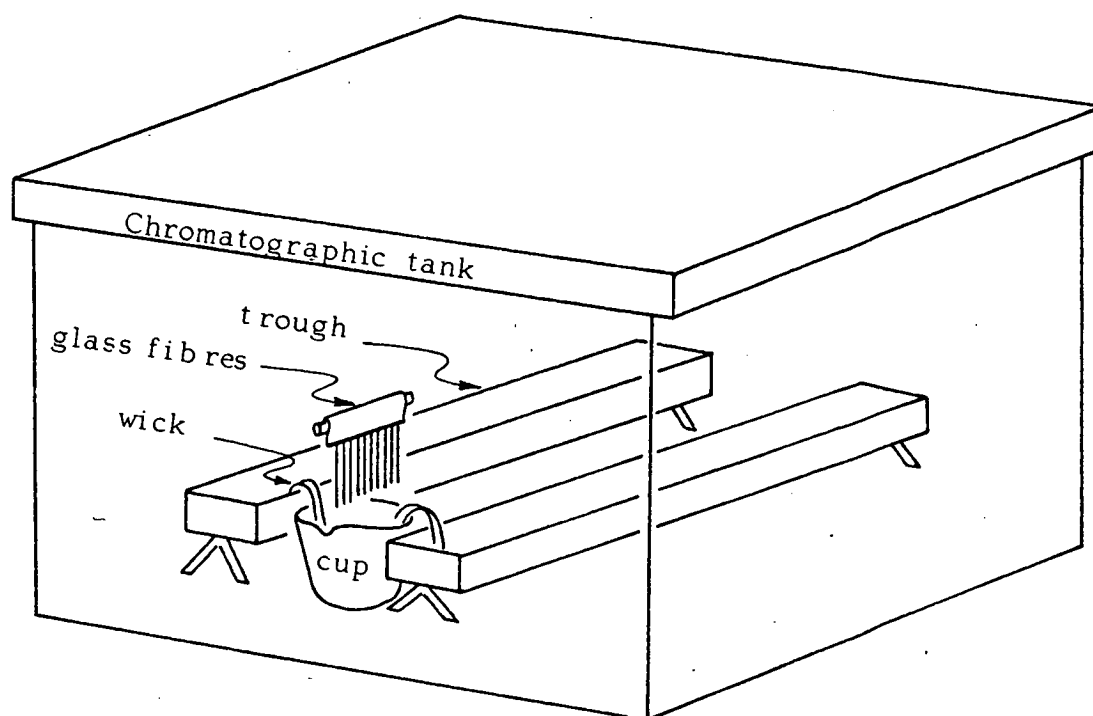
fixed with adhesive tape to a short glass rod – 10 per rod. Cups of 25ml capacity were placed between two glass troughs enclosed in a chromatographic tank. The troughs were filled either with 0.1M CaCl_2 (pH 6.4) or 0.1M sodium oxalate (pH 6.4). The cups were filled with 19ml of 0.2M sodium acetate buffer (pH 6.1) and 1ml of the test solution. Wicks (6mm x 11cm) were cut from Whatman no. 1 paper and placed two per cup so that one led to the calcium solution, the other to the oxalate solution. The experimental apparatus is illustrated in Figure 4.1.

At specified times wicks were removed, the glass rods taken out and either examined under a light microscope or analysed for calcium, essentially by the method of Kepner and Hercules (1963). For calcium analysis the glass fibres were left overnight in 4ml. of 2N HCl before 10-20 μl was removed from solution and added to a calcein solution (0.7mg/100ml 0.8N KOH) in test tubes washed in 1N HNO_3 . After 10-20 min samples were read in a fluorimeter (excitation 360nm, primary filter Schott UG11; emission >410nm, secondary filter Wratten 2A) and compared with standards of 0-12mg% made from anhydrous CaCO_3 .

4.3.2.2 Metastable solution method

Seed crystals were added to a metastable solution of calcium oxalate as described by Meyer and Smith (1975a). The seed crystals were prepared by adding dropwise over 2h solutions of 100ml of 0.4M CaCl_2 and 100ml of 0.4M potassium oxalate to 300ml of distilled water kept at 70°C. After this addition, the seed crystals were maintained at 70°C for a further 5h then allowed to cool overnight to room

Figure 4.1 Apparatus used to measure growth rate of calcium oxalate crystals on glass fibres*.



*The equipment used was essentially that described by Sallis and Lumley (1979).

temperature. The solid was collected by centrifugation and repeatedly washed with distilled water until the supernatant gave a negative test for chloride (addition of conc. AgNO_3 to the supernatant). The solid was thoroughly dried then suspended in water (18mg/ml). Sufficient stock solution for subsequent experiments was retained and incubated for at least 10 days at 37°C to mature the seed crystals.

In order to perform the assay, 0.5ml of 50mM CaCl_2 and 0.5ml of 50mM potassium oxalate were added to 49ml of 0.15M NaCl at 37°C and stirred mechanically. 1.0ml of the test solution was added and the pH adjusted to 6.0. Then 0.4ml of the seed crystals suspension was added, the pH checked and a sample immediately taken and filtered through a Millipore filter ($0.45\ \mu\text{m}$). Samples (2.0ml) were removed at 0, 5, 10, 15, 30, 45, 60 and 75 min, and filtered through a Millipore membrane ($0.45\ \mu\text{m}$).

Calcium in the filtrates was determined by Atomic Absorption Spectroscopy. Lanthanum solution (0.4ml of 50,000 ppm), 5N HClO_4 (0.2ml) and water (0.7ml) were added to samples (0.7ml) and thoroughly mixed. Concentrations were determined on a scale calibrated between 2 and $8\ \mu\text{g Ca}^{++}/\text{ml}$.

4.3.3 ADSORPTION OF PC ONTO HAP AND CALCIUM OXALATE

250mg of either calcium oxalate or HAP was added to $193 \pm 10\ \mu\text{g}$ citrate (present as PC) in 3ml of 0.15M NaCl at 37°C and pH 7.4. After brief stirring, the solid was spun down and the supernatant analysed for free and total citrate.

4.4 RESULTS

4.4.1 CALCIUM PHOSPHATE CRYSTALLISATION

4.4.1.1 Comparison of the anti-calcifying ability of PC

Figure 4.2 details the amount of base added in order to maintain a constant pH in suspensions of calcium phosphate formed in the presence or absence of test compounds. The break in the rate of base addition corresponds to the ACP to HAP transition (Boskey and Posner, 1973) and a compound inhibits if it delays the onset of this transition (I_t). Clearly, as shown in the figure, although ATP and EHDP are both inhibitors, 50 μ M of EHDP is more effective than 40 μ M ATP. The relative potency of compounds can be better assessed if a comparison is made of the effect of inhibitors over a given concentration range on I_t . Thus Figure 4.3 compares PC with some selected inhibitors and indicates that their potency is in the order: PC >> EHDP > ATP >> citrate. Not only does PC inhibit, but it is the most potent inhibitor of the compounds tested. Notably EGTA, a chelator with a high affinity for calcium affinity, did not inhibit even at 0.5mM.

4.4.1.2 Interaction of PC, citrate and Mg^{++} ; and PC with an artificial urine

Under physiological conditions it is unrealistic to envisage one inhibitor acting in isolation. In order to gain some information on how PC might act in vivo, the interactions between Mg^{++} , citrate and PC were examined. These experiments at least indicate whether interactions are possible,

Figure 4.2 Inhibitory effect of ATP and EHDP on the transition of ACP into HAP.

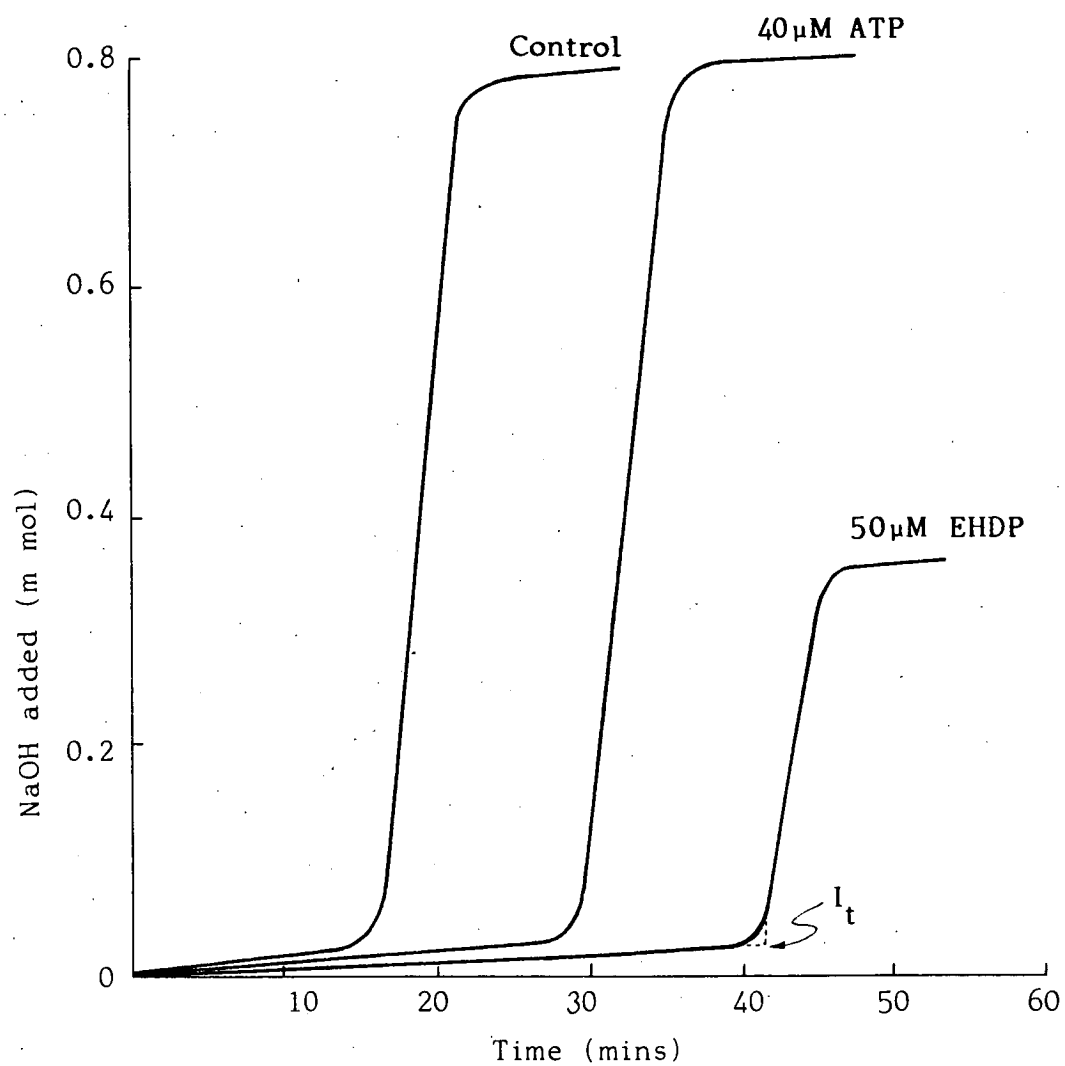
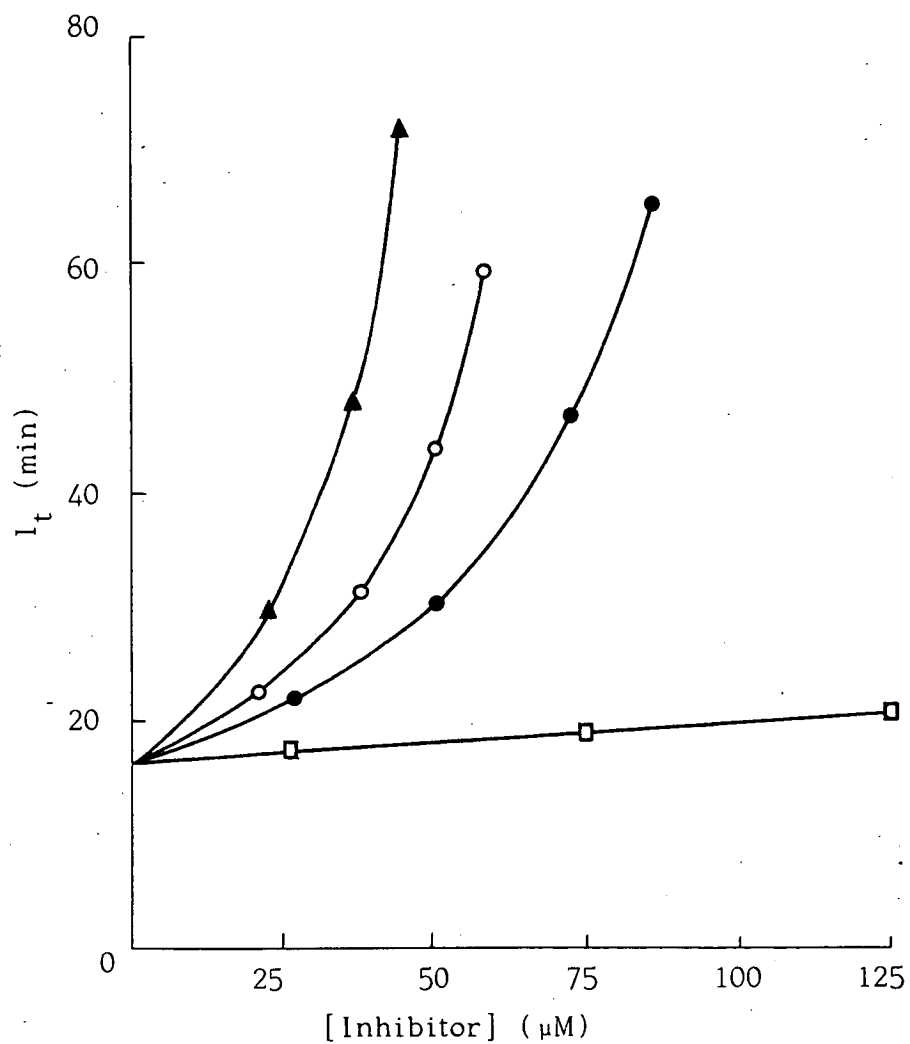


Figure 4.3 Effect of inhibitor concentration on the onset of the transition (I_t) of ACP into HAP.



▲ — ▲ PC

● — ● ATP

○ — ○ EHDP

□ — □ citrate

although the physiological state is not closely modelled. Results from these experiments are given in Table 4.1. Tests were conducted at 20°C to emphasise interactions, since at 37°C induction times are decreased. PC with either citrate or Mg^{++} acts synergistically, but in contrast Mg^{++} and citrate do not inhibit to the extent expected on the basis of an additive effect on inhibition. The three inhibitors act together synergistically but not to the extent expected, considering the action of Mg^{++} and PC; and PC and citrate.

These preliminary results indicated that PC could perhaps interact with ions present in physiological fluids. Consequently the action of PC in the presence or absence of an artificial urine was investigated. The synthetic urine used was that described by Miller et al. (1977) and its constituents are given in Table 4.2. PC (20 μ M) gives an increase in the induction time of 25min and 5ml of synthetic urine gives an increase in the induction time of 6min. Together an increase in I_t of 56min results, corresponding to a 180% increase over an additive effect on inhibition.

4.4.2 CALCIUM OXALATE PRECIPITATION

4.4.2.1 Glass fibre system

Table 4.3 gives the abilities of pyrophosphate, PC and chondroitin sulphate C [MW ca. 60,000 (Yamagata et al., 1968; Schiller et al., 1961)] to decrease the deposition of calcium oxalate onto glass fibres. Data indicate that chondroitin sulphate C is a more potent inhibitor than PC on a molar basis; weight-for-weight PC is much more effective.

In the absence of added inhibitors, crystals on the glass fibres were hexagonal deposits characteristic of the

Table 4.1. Inhibitory ability resulting from interactions of Mg^{2+} , PC and citrate on HAP formation.

COMBINATION*	$\Delta I_t(\text{min})^\dagger$	$\Delta I_t(\text{exp.})^\ddagger$	DIFFERENCE (min) [$\Delta I_t - \Delta t_t(\text{exp.})$]	SYNERGISM
PC	15	-	-	-
Mg^{2+}	12	-	-	-
citrate	7	-	-	-
PC + Mg^{2+}	49	27	+22	YES
PC + cit.	59	22	+37	YES
Mg^{2+} + cit.	16	19	-3	NO
PC + Mg^{2+} + cit.	50	34	+16	YES

*Amounts used were: 3.0mg PC, 28mg $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$ and 28mg trisodium citrate dihydrate.

$^\dagger I_t$'s were determined at pH 7.4, 20°C , as described in the text.

$^\ddagger \Delta I_t(\text{expected})$ was calculated on the basis of an additive effect of inhibitors on HAP formation.

Table 4.2. Composition of an artificial urine.

SUBSTANCE	AMOUNT (g/l)
Na_2SO_4	3.33
KH_2Cl	3.18
KCl	8.31
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	2.00
Na_2HPO_4	0.58
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	4.12
NaCl	9.25
$\text{Na}_3 \text{ citrate} \cdot 2\text{H}_2\text{O}$	0.80

Taken from Miller et al. (1977).

Table 4.3. The ability of PC, pyrophosphate and chondroitin sulphate C to inhibit calcium oxalate crystal formation on glass fibres.

COMPOUND	[INHIBITOR] (mM)	% INHIBITION*
Pyrophosphate	1.1	48 ⁺⁴
PC	0.33	66 ⁺³
Chondroitin sulphate C	0.10	55 ⁺⁷

*3 trials; mean ⁺¹ S.D.

Table 4.4. Adsorption of PC onto calcium oxalate and HAP.

ADSORBENT TYPE (250mg)	[PC] _{initial} (μg)*	[PC] _{final} (μg)*	%ADSORPTION
HAP	193 ⁺¹⁰	1.1 ^{+0.2}	99
Calcium oxalate	193 ⁺¹⁰	105 ⁺⁴	46

*4 trials; mean ⁺¹ S.D.

dihydrate. Pyrophosphate presence altered the crystal habit to dodecahedrons of the dihydrate. In the presence of PC or chondroitin sulphate C, crystals were predominantly of the oval, biscuit and dumb-bell habits characteristic of the monohydrate, although some dodecahedral prisms were also observed.

4.4.2.2 Metastable solution method

4.4.2.2.1 Comparison of PC with pyrophosphate

In order to confirm results from the glass fibre system suggesting PC was more potent than pyrophosphate as an inhibitor of calcium oxalate precipitation, comparative experiments were undertaken using the metastable solution method. Figure 4.4 shows the rate of depletion of calcium from solution upon seeding a metastable solution with calcium oxalate crystals with or without 27 μM inhibitor. The ion-pairing correction outlined by Meyer and Smith (1975a, b) is not necessary in these cases due to the small amount of inhibitor added. Clearly, PC inhibits more than pyrophosphate (PP_i) in this system. For quantitation, the data were treated as described by Meyer and Smith (1975b); Figure 4.5 indicates that 27 μM PP inhibits 50% and 27 μM PC, 82%, substantiating the results obtained with the glass fibre system.

4.4.2.2.2 Interactions of PC, Mg^{++} and citrate

In order to investigate whether synergism between inhibitors occurs in the precipitation of calcium oxalate, experiments similar to those conducted in the calcium phosphate system were undertaken. However, with the calcium oxalate system, if sufficient ions were added

Figure 4.4 Effect of inhibitors on the rate of depletion of calcium from a seeded metastable solution of calcium oxalate.

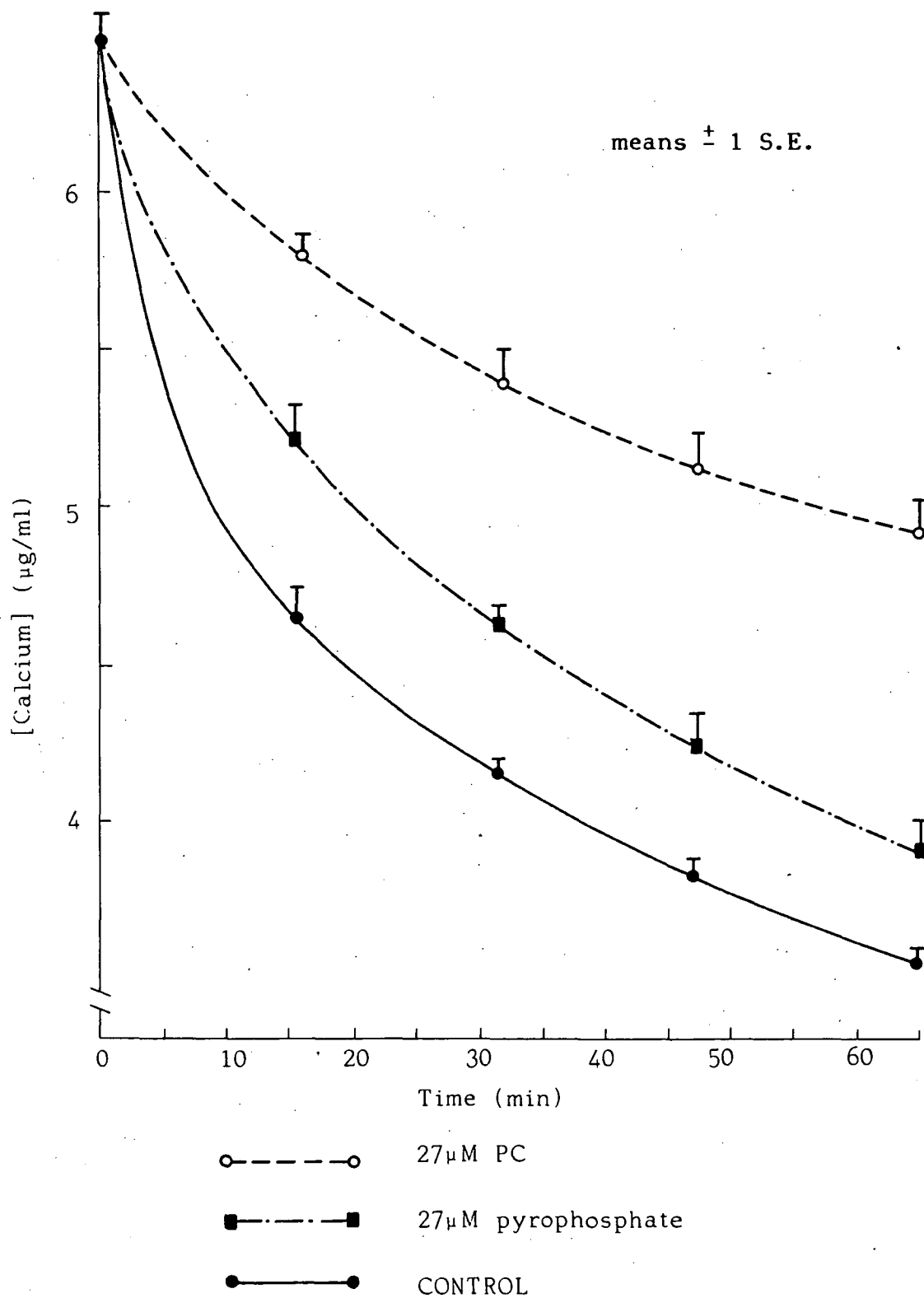
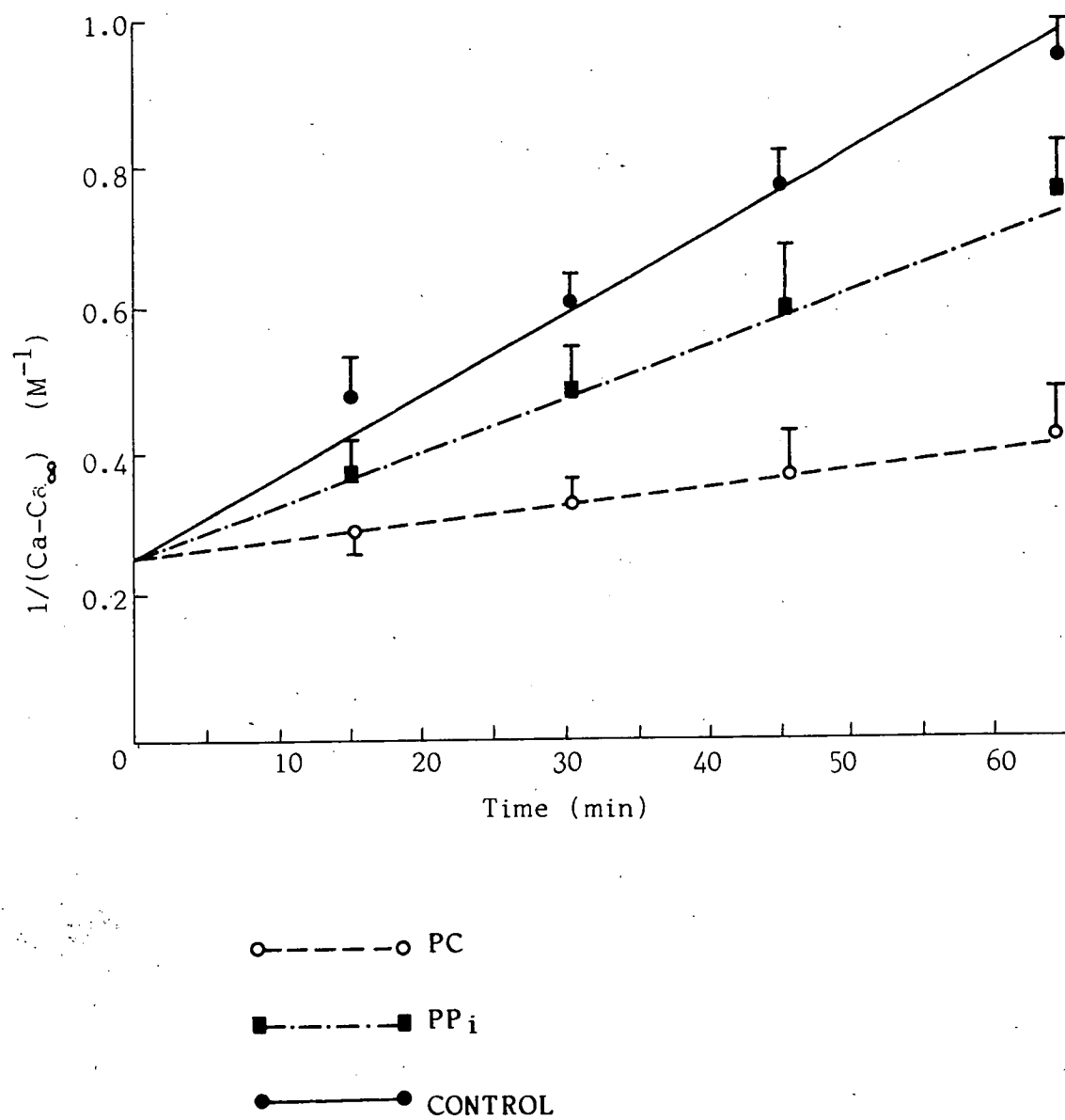


Figure 4.5 Quantitation of the inhibitor potency of PC and pyrophosphate.



into the seeding assay, which interact with Ca^{++} or oxalate, relatively elaborate computation must be carried out to correct for ion-pairing. If two ions are added the computation is complicated. For example, if Mg and citrate are added, then the following interactions must be considered: citrate with Mg^{++} and Ca^{++} , and oxalate with Mg^{++} and Ca^{++} . Further, allowance must be made for differing affinities of ion-pairing. These difficulties can be avoided by adding small amounts of Mg^{++} and citrate sufficient to warrant computational correction. Under these conditions no synergism with PC was observable as shown in Figure 4.6.

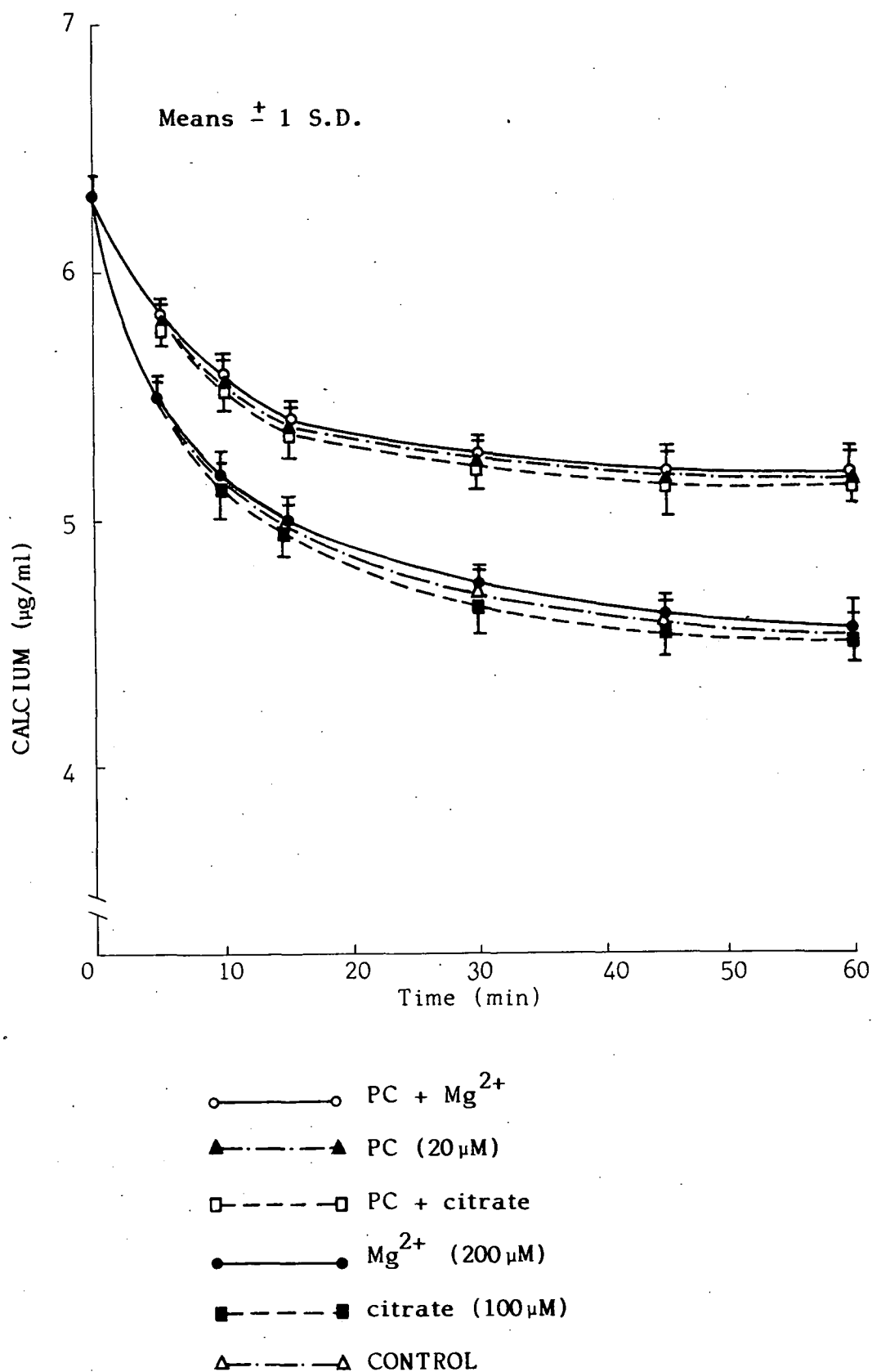
4.4.3 ADSORPTION OF PC ONTO CALCIUM OXALATE AND HAP

Adsorption of inhibitors onto calcium salt surfaces has been considered to be the likely mechanism of inhibition. Active growth sites are blocked decreasing possibilities for future crystal growth and nucleation. Investigation of the possible adsorption of PC onto calcium oxalate and HAP was therefore undertaken. Table 4.4 gives the amounts of PC left in solution after the addition of either calcium oxalate or HAP: 250mg of HAP adsorbed > 99% of the initial PC in solution, whereas 250mg of calcium oxalate absorbed 46%. In both cases the remaining solution contained <4% free citrate. These findings conform to the idea that inhibitors act by being adsorbed onto active growth sites.

4.5 DISCUSSION

The data presented here indicate PC is a potent inhibitor of both calcium phosphate and calcium oxalate crystallisation and does not require the presence of citrate

Figure 4.6 The effect of adding Mg^{2+} , citrate or PC alone or in combination on the rate of depletion of calcium from a seeded metastable solution of calcium oxalate



for inhibition, contrary to the report of Tew and Mahle (1977). In fact, PC is significantly more powerful as an inhibitor of HAP formation than EHDP and ATP, the latter two compounds being recognised as two of the most potent inhibitors (Fleisch, 1978). The finding of this remarkable inhibitory ability of PC indicates it could play a role as an inhibitor in biological systems and therefore gives some support to the suggestion that PC may be the factor responsible for the potent inhibition shown by various biological fractions (Howard, 1976; Lehninger, 1977). However, before the inhibitory effect of these fractions is attributed to PC, careful purification is required to demonstrate that PC is present and is the effective factor.

PC inhibits HAP formation although it does not contain "two ester phosphates" which have been suggested to be structural prerequisites for inhibition; this indicates a reappraisal of the structural factors that affect inhibition is necessary. The testing of a wide range of compounds for inhibitory ability that will enable elucidation of the structure-ability relationship of inhibitors is indicated.

As to the mechanism(s) by which PC inhibits HAP formation, inhibition cannot simply be due to Ca^{++} chelation as Sutor and Percival (1978) have suggested, since EGTA does not significantly affect the transformation process. The strong adsorption of PC onto HAP indicates that binding to active growth sites is the likely mechanism consistent with proposals by Meyer and Nancollas (1973), and Meyer et al. (1974) on the action of other phosphoric ester inhibitors.

The mechanism of action of a single ion is readily explicable but the underlying mechanism(s) responsible for the synergistic interactions between ions are more difficult to discern. It appears likely that the reason Mg^{++} and PC interact synergistically is analogous to that proposed for the synergism between Mg^{++} and ATP: chelation of Mg^{++} and PC protects PC from transformation that is known to occur on calcium phosphate surfaces (Krane and Glimcher, 1962). In contrast, Mg^{++} and citrate combined causes a decrease in expected inhibition, perhaps due to a reduction in concentration of their active species by chelation. The basis for the synergism between citrate and PC is not obvious from the results presented here, but a likely explanation is that inhibitors bind to different sites on calcium phosphate surfaces so that they efficiently cover the surfaces, blocking growth and seeding. When all three inhibitors - Mg^{++} , citrate, PC - are present, synergism is still apparent, although less than that expected on the basis of the Mg^{++} /PC and PC/citrate couples. Presumably this is due to the Mg^{++} /citrate interaction which decreases the amount of free Mg^{++} and citrate available for synergism with PC. Synergism was also observed between PC and an artificial urine suggesting that in vivo PC could act as a potentiating agent, a small concentration serving to greatly increase the anti-calcifying ability of a particular biological fluid. It should be noted, however, that although such physico-chemical phenomena are prominent in well-defined in vitro calcifying systems, it may not be valid to suggest that therefore this occurs physiologically.

Tew et al. (1978) have suggested that mitochondria

contain PC on the basis that mitochondrial extracts inhibit the transformation of ACP into HAP. However, since many compounds inhibit this fraction (Termine and Conn, 1976; Termine and Posner, 1970), this result is equivocal and direct proof, involving isolation and characterisation of the active ingredient, is required.

PC is a potent inhibitor not only of HAP formation but also of calcium oxalate formation. In fact, weight-for-weight PC is the most potent of the inhibitors tested which included chondroitin sulphate C, accepted to be one of the most potent inhibitors of calcium oxalate formation. However, on a molar basis, chondroitin sulphate C is more powerful than PC.

PC affects the morphology of calcium oxalate crystals which grow on glass fibres suspended in a solution of Ca^{++} and oxalate. Without inhibitors, deposits were hexagonal deposits characteristic of the dihydrate (weddelite), whereas in the presence of PC or chondroitin sulphate C crystals were predominantly of the oval, biscuit or dumb-bell habits, characterising the deposit as the monohydrate (whewellite). These observations concur with previous reports (Sutor, 1969; Welshman and McGeown, 1972) indicating inhibitors alter crystal habit.

The potent ability of PC as an inhibitor of calcium oxalate precipitation was confirmed with an assay which used calcium oxalate seed crystals to initiate deposition from a metastable Ca^{++} and oxalate solution. In contrast to the synergism between PC, citrate and Mg^{++} observed in the inhibition of HAP formation, no synergism was detectable

with calcium oxalate precipitation.

The mechanism by which PC inhibits calcium oxalate precipitation is likely to be adsorption of PC onto growth sites as indicated by the ability of calcium oxalate to adsorb PC. Once blocked, these sites can no longer nucleate further growth, so a decrease in the number of active sites inhibits crystal growth.

PC then has been demonstrated to be a potent inhibitor of both calcium phosphate and calcium oxalate formation. Indeed, with regard to calcium phosphate precipitation, the synergistic interaction between PC and other ions, as demonstrated here, suggests that PC could act as a potentiating agent, amplifying the inhibitory potential of a group of inhibitors. The physico-chemical characteristics indicate that if PC is found naturally then it could be a new inhibitor for consideration in the control of biological calcification. This work emphasises the need to define unambiguously the biological occurrence of PC with a view to clarifying its sites of action and therefore possible biological roles.

4.6 SUMMARY

1. PC was found to be a potent inhibitor of HAP formation; it was demonstrated to be more powerful than ATP, EHDP and pyrophosphate.
2. PC interacts synergistically with citrate and Mg^{++} to inhibit HAP but not calcium oxalate formation.
3. PC potentiates the ability of an artificial urine to inhibit HAP formation.
4. PC potently inhibits calcium oxalate formation.

5. Using a glass fibre nucleating system, PC was found to modify the crystal morphology, and to be more potent - on a weight-for-weight basis - than pyrophosphate or chondroitin sulphate C.

6. Using a method involving the seeding of a metastable solution of calcium and oxalate, PC was found to be a more potent inhibitor than pyrophosphate. No synergism between Mg^{++} , citrate and pyrophosphate was found.

7. PC was adsorbed onto calcium oxalate and HAP.

CHAPTER 5

STRUCTURE-ACTIVITY RELATIONSHIP OF INHIBITORS OF HYDROXYAPATITE PRECIPITATION

5.1 INTRODUCTION

Termine and Conn (1976) have suggested that low molecular weight compounds will inhibit the transformation of amorphous calcium phosphate (ACP) into hydroxyapatite (HAP) if they possess at least two ester phosphates. However, the demonstration that PC potently inhibits indicates that this criterion requires re-appraisal since PC is a monophosphate. In order to define the structural factors influencing inhibitor power, a selection of compounds was tested for inhibitory activity and this chapter reports on the results of the study. Two of the compounds examined were carboxylic monophosphates, phosphonoacetate and phosphonoformate, which, along with PC, do not conform to the structural criteria proposed by Termine and Conn (1976).

The development of a structure-activity relationship of inhibitors of HAP formation could be of use in several areas, having the potential to aid:

1. the identification of biological compounds which on structural considerations are predicted to inhibit, but are as yet unrecognised;
2. the understanding of the nature of the binding sites and the mechanism of binding to calcium phosphate surfaces, and
3. the prediction of structures of inhibitors that might

be worth synthesizing for use in the therapy of some Ca^{++} metabolic diseases. [These compounds should inhibit HAP precipitation, so the Ca^{++} metabolic diseases that could be treated include Paget's disease, urolithiasis and osteolytic sarcoma.]

Although the structure of inhibitors may yield some information on the nature of the binding sites, further data on the mechanism(s) of inhibition was sought by monitoring the transformation of ACP into HAP not only by the usual base titrimetric technique but by other methods. These methods involved measurement of calcium ion activity and of laser light scattering. During the course of this study it became evident that data obtained from light scattering measurements added a further dimension to the present study since it was possible to separate the inhibitors into two distinct groups. Observation by transmission electron microscopy (TEM), together with other data on the conversion process, confirm this division and have enabled consideration of the mechanism(s) of inhibition responsible for the grouping of the inhibitors.

A synergistic interaction between Mg^{++} and PC, and between citrate and PC on the inhibition of HAP formation were noted in the preceding chapter. To clarify the mechanism(s) of such interactions, and also to examine whether other interactions of this nature might occur in vivo, the activity of pairs of inhibitors was studied with particular reference to the two groups separated by the light scattering data.

The findings presented here and the results and proposals of other workers which directly or indirectly

substantiate the conclusions reached, are discussed in terms of the structure-activity relationship of inhibitors and the secondary factors influencing inhibitor power.

5.2 METHODS

5.2.1 MONITORING OF THE CONVERSION PROCESS

Monitoring of the conversion process was achieved by measuring three parameters:

a. Base titration

The base titrimetric method was essentially that described by Meyer and Eanes (1978a), outlined in the preceding chapter (4.3.1).

b. Calcium-ion activity

A calcium-ion selective electrode (Type F2112Ca) and a reference electrode (Type 90-01) from Radiometer, Copenhagen, Denmark, were immersed in the stirred suspension. The system was calibrated with Ca^{++} -NFTA buffers from 5×10^{-5} to 5×10^{-3} mM Ca^{++} enabling direct determination of ion activity without computation.

c. Turbidity

A laser light beam (laser model KED-1, 633nm from Laser Electronics, Queensland, Australia) was directed through the suspensions to impinge on a cadmium sulphide light dependent resistor (ORP 12) with a peak spectral response of 610nm (Radiospares Components, Subiaco, Western Australia). Signals from both the detector and calcium-ion selective electrode were fed through a voltage amplifier and outputs were coupled to a two-pen 10mV potentiometric recorder. The turbidimetric system was calibrated with suitable filters. Variation

in background light intensity, which initially led to instability in the traces, was eliminated by masking the sides of the water bath with black plastic. The use of laser light for turbidity measurements eliminates problems with UV light requiring monitoring in stirred cuvettes (Termine and Eanes, 1972), whereby turbidity and base titration could not be simultaneously monitored as the mixture required strong buffering with Tris.

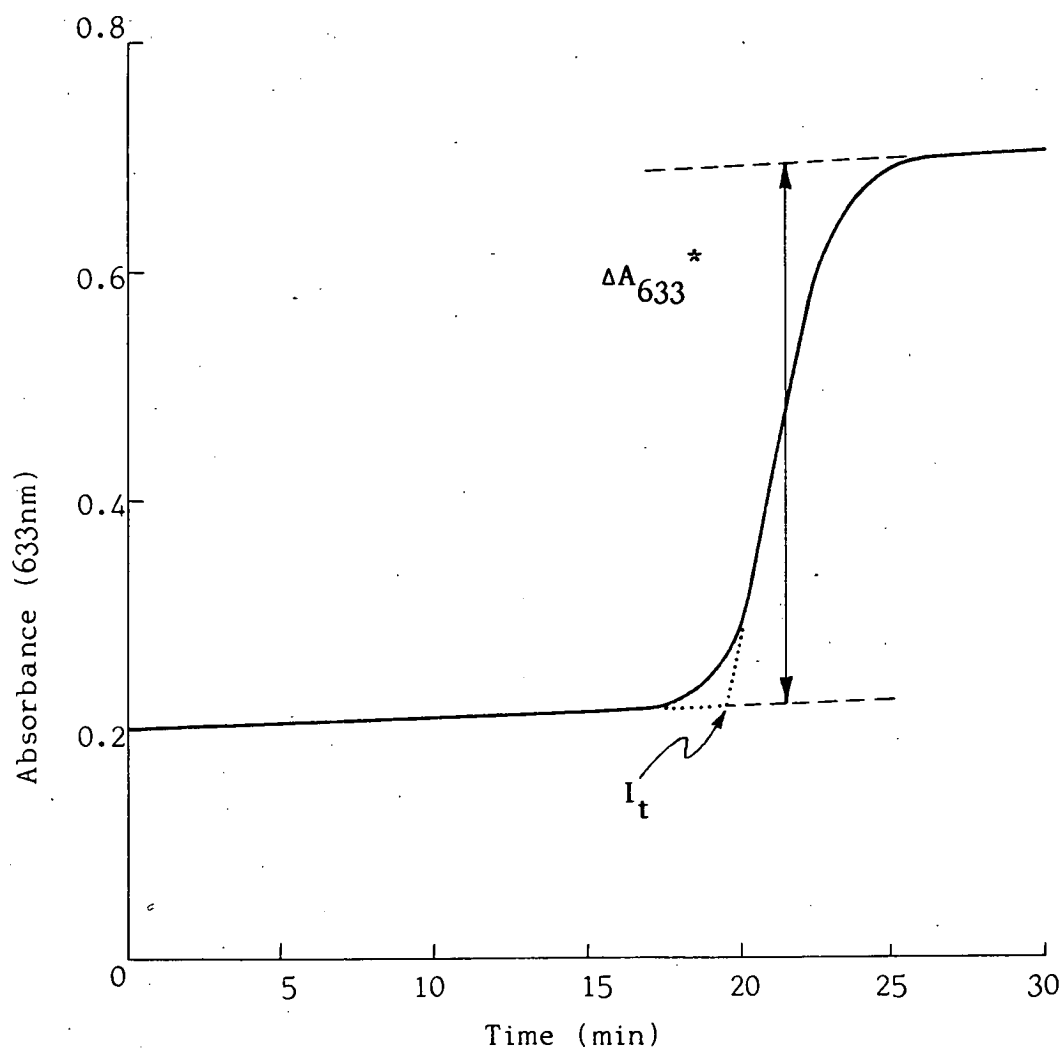
Figure 5.1 illustrates the method used to calculate data. An upward deflection of the pen represents increasing turbidity associated with a change of ACP into HAP. Tangents were drawn to the flattest part of the traces representing the pre- and post-transformation period as shown by the dotted lines. The differences between these two values was designated ΔA_{633} . Data from base titrimetric and calcium-ion activity measurements were calculated in an identical fashion.

The precise time at which the conversion process starts (i.e. the induction time: I_t) was determined as described by Meyer and Eanes (1978a) and, as illustrated in Figure 5.1, it is the point of intersection of the tangents to the flattest portion of the pre-transition period and the steepest part of the transformation period. The value for I_t could be calculated from any of the three parameters monitored since they changed simultaneously.

5.2.2 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Prior to and during the transformation process, aliquots of the reaction mixture were removed for TEM analysis. Drop-lets were placed on formvar-coated grids and excess liquid

Figure 5.1 Change in turbidity with time and the method of calculating the induction time (I_t).



* ΔA_{633} is the change in turbidity associated with the conversion process.

removed after 30s by touching the edges with filter paper (Eanes et al., 1976). Following air-drying the specimens were examined in an Hitachi H300 electron microscope at 75kV. In some instances samples removed after the transition were filtered (Millipore membrane, 0.45 μ m) and the solid embedded in Epon before sectioning (Francis, 1969).

5.3 RESULTS

In the preceding chapter it was noted that a plot of I_t against inhibitor concentration indicated that as inhibitors PC \gg ATP $>$ EHDP $>$ citrate. Notably, although citric acid was a very weak inhibitor, the incorporation of a phosphate group into the molecule in PC produced powerful inhibition. Figure 5.2 shows the inhibitory ability of PNP, PP_i and phosphonoformate; they all inhibit indicating that multiple carboxyl groups, whilst enhancing inhibitory ability, are not the minimum requirement for potent inhibition. Any compound with a phosphate group and an acidic group, either a carboxylic or phosphate group, for example phosphonoformate, will potently inhibit.

To determine the effect of inhibitor power of the proximity of relevant groups, the potency of phosphonoformate, 2-phosphoglycerate and 3-phosphoglycerate were compared as shown in Figure 5.3. Data show that the shorter the bonding between phosphate and carboxylic groups, the more powerful the inhibitor is since PF $>$ 2-PG $>$ 3-PG. In other experiments compounds such as o-phosphoserine and o-phosphothreonine which were tested to investigate the effect of amino group substitution, failed to inhibit over the same concentration

Figure 5.2 Inhibitory ability of imidodiphosphate (PNP), pyrophosphate (PP_i) or phosphonoformate (PF) over a concentration range.

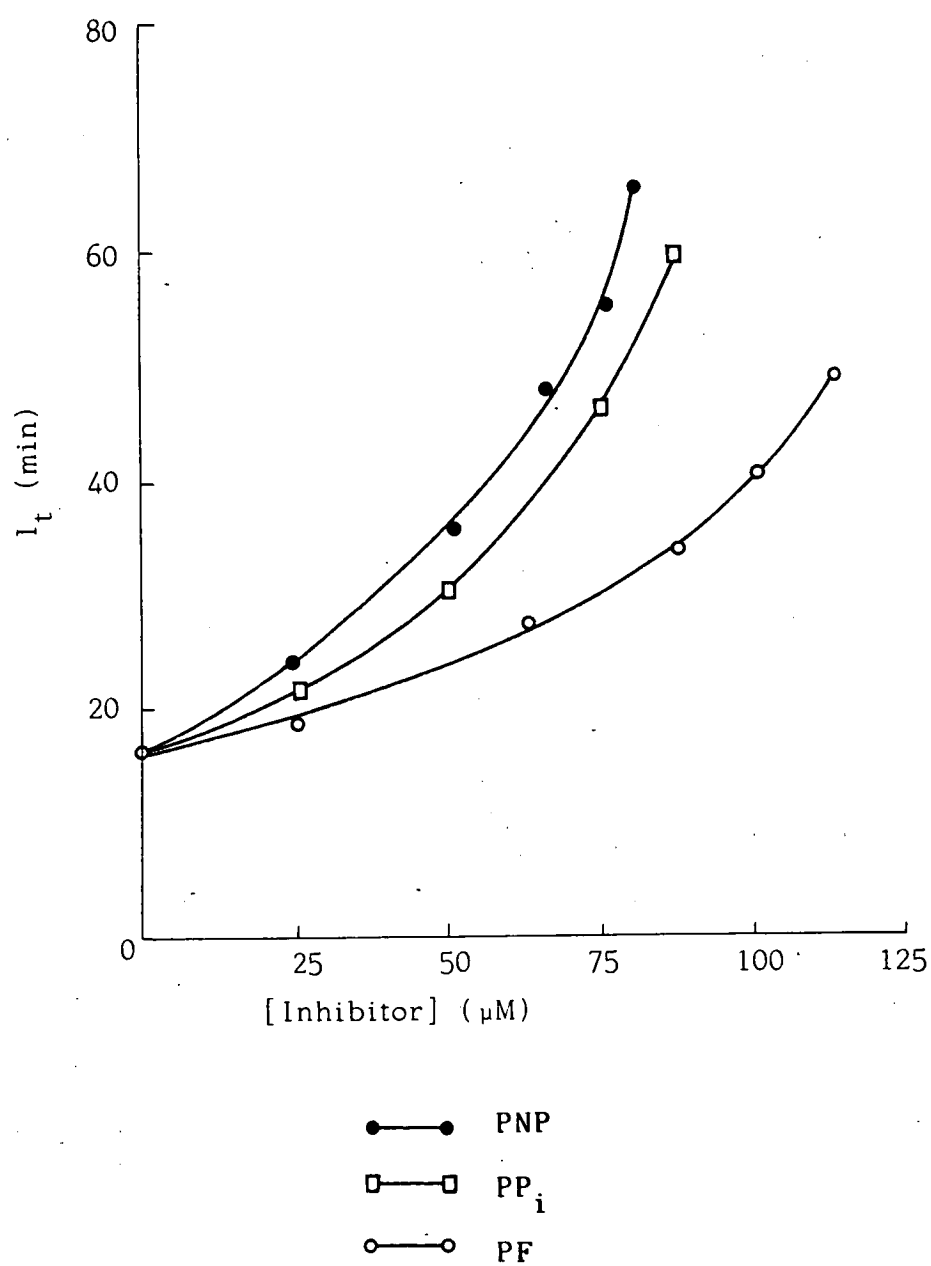
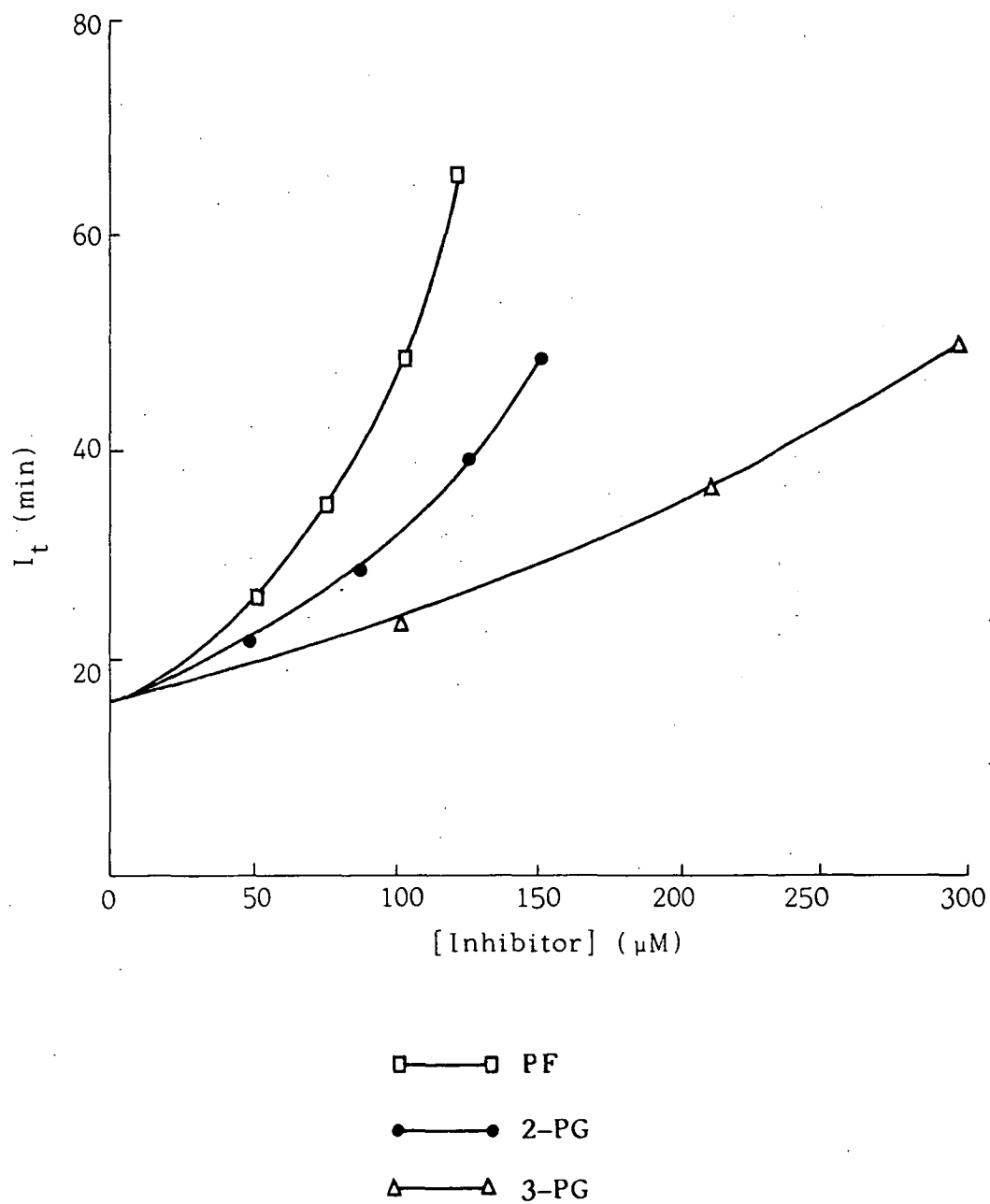


Figure 5.3 Effect of increasing concentrations of phosphonoformate (PF), 3-phosphoglycerate (3-PG) and 2-phosphoglycerate (2-PG) on I_t .

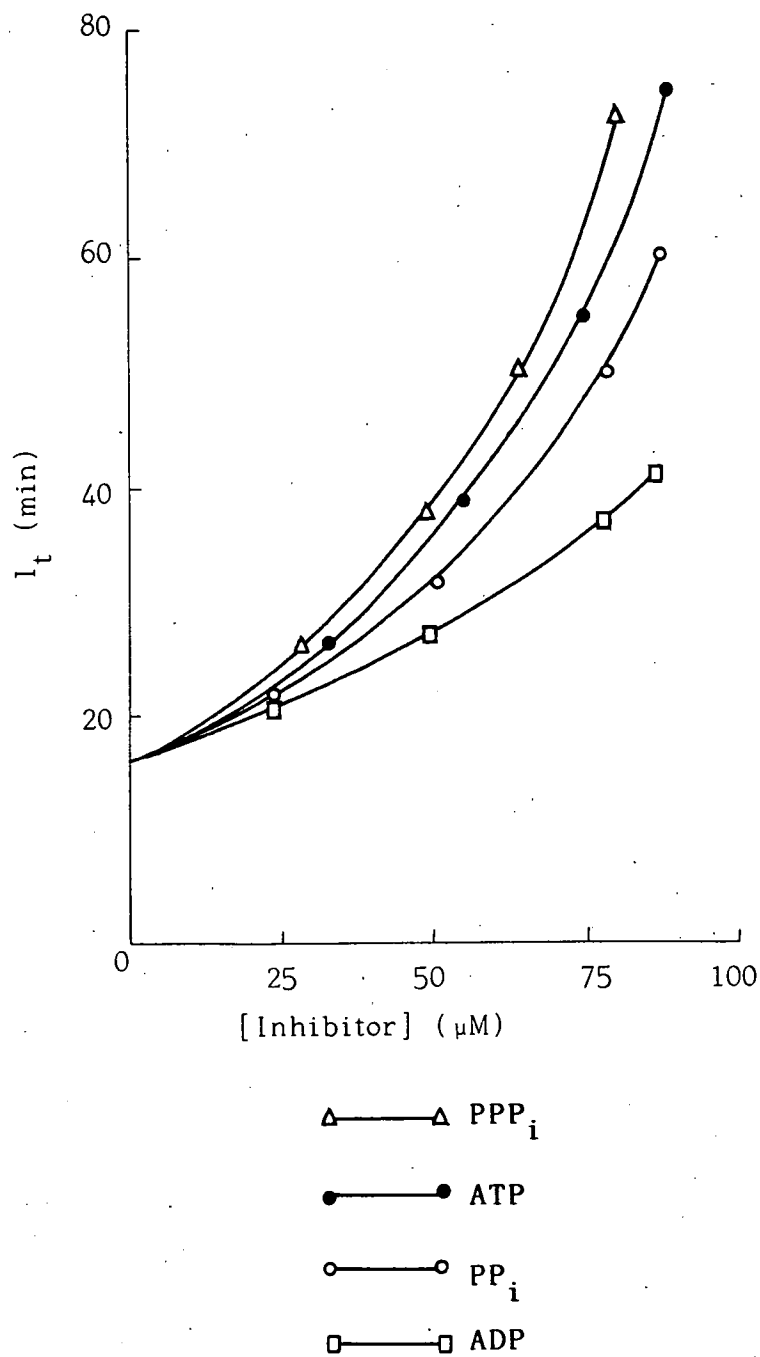


range. Similarly, no inhibition occurred with fructose 1-phosphate, EGTA, *p*-nitrophenyl phosphate and the carboxylic acids: formic, succinic, malic and oxalic acids.

The effect of substituting sterically bulky groups onto an inhibitor, and the influence of the number of active groups present, was examined using ATP, ADP, tripolyphosphate and PP_i . The results are shown in Figure 5.4 indicating that inhibitor power increases with the number of active groups, in this instance phosphate groups. The sterically bulky adenosine moiety decreases inhibitor potency since ATP is not as powerful as tripolyphosphate nor ATP as PP_i . To evaluate the possibility that the bond angle between the active groups modulates inhibitor power, phosphoenol pyruvate was compared with phosphonoacetate at particular concentrations. At 50 μ M phosphonoacetate inhibited but no effect was seen with phosphoenol pyruvate - even at 300 μ M.

In order to further investigate the effect of an inhibitor on the precipitation of ACP and its subsequent transformation into HAP, the magnitude of base uptake, calcium-ion depletion and turbidity change (ΔA_{633}) associated with the transition was compared over a range of inhibitor concentrations. It is evident from the base uptake curves alone that inhibitors affect the conversion process in either of two ways. As shown in the preceding chapter (see Figure 4.3, Section 4.4.1.1), ATP does not alter the amount of base consumed during the transformation, whereas EHDP causes a decrease in base consumption. It is data from the effect of inhibitor concentration on ΔA_{633} however, that more definitively separate inhibitors into two classes which are referred

Figure 5.4 Effectiveness of ATP, ADP, tripolyphosphate (PPP_i) and pyrophosphate (PP_i) as inhibitors of HAP formation: influence of the number of P_i groups on inhibitory power and modification by the presence of adenosine



to here as Type I and Type II inhibitors. Typical results are shown in Figure 5.5 for a Type I inhibitor (EHDP) and indicate that the magnitude of the turbidity change (ΔA_{633}) reaches a maximum at a particular concentration (for example, with EHDP it is 40 μ M). At higher concentrations ΔA_{633} rapidly decreases concomitant with a rapid reduction in base uptake and calcium-ion depletion from solution. Figure 5.6 gives typical results for a Type II inhibitor (ATP) indicating that in contrast to Type I inhibitors they do not affect ΔA_{633} , the amount of base uptake nor calcium-ion depletion. A range of inhibitors has been tested in this fashion and, when data are plotted for each inhibitor, graphs take either of the two forms shown in Figures 5.5 and 5.6, thus enabling unambiguous grouping of the compounds.

Selected data from such groups are tabulated in Tables 5.1 and 5.2. For Type I inhibitors the concentration eliciting the maximum ΔA_{633} is given in Table 5.1, together with a higher and lower concentration for contrast. The effect of these concentrations on the amount of base uptake, Ca^{++} -ion depletion and I_t is also given. With Type II inhibitors, representative concentrations over a broad range are tabulated in Table 5.2 which gives their effect to delay the onset of the transformation (i.e. the induction time, I_t). Type I inhibitors then, include (in order of potency): PC, EHDP, tripolyphosphate, imidodiphosphate and PP_i . Type II inhibitors include: ATP, ADP, phosphonoformate, 2-phosphoglycerate, 3-phosphoglycerate, Mg^{++} and citrate.

The morphology of the calcium phosphate particles formed in the presence or absence of inhibitors from either

Figure 5.5 Effect of EHDP concentration on the transformation of ACP into HAP.

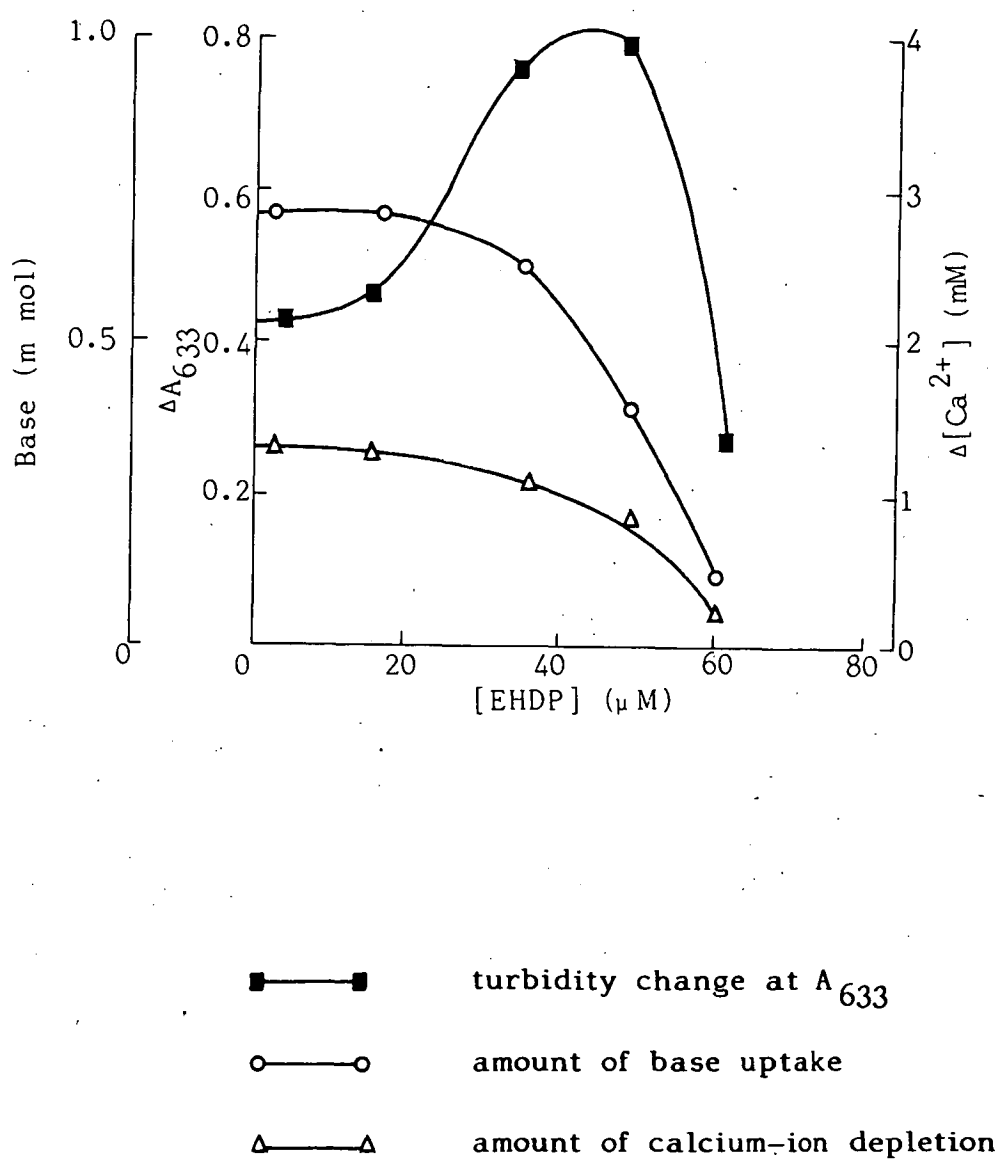


Figure 5.6 Effect of ATP concentration on the transformation of ACP into HAP.

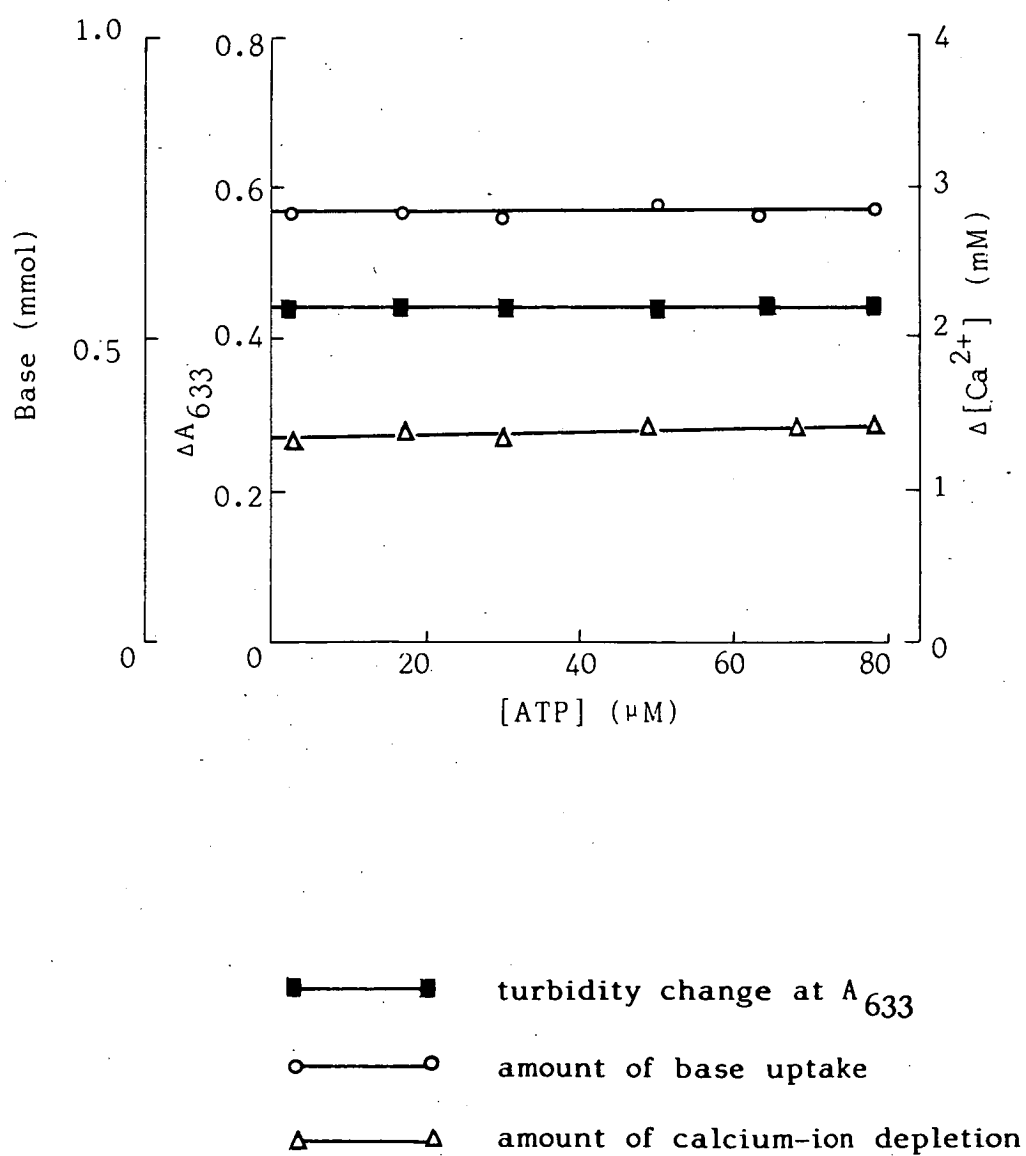


Table 5.1 Effect of increasing concentration of Type I inhibitors on the parameters defining the ACP to HAP transformation.

INHIBITOR	[INHIBITOR] (μM)	ΔA_{633}	ΔCa^{++} (mM)	ΔOH (mM)	I_t (min)
None	-	0.44	1.3	0.75	17
PC	10	0.60	1.3	0.74	22
	25	0.96	1.2	0.70	35
	40	0.45	0.3	0.35	65
EHDP	15	0.50	1.3	0.75	22
	40	0.80	1.0	0.70	32
	55	0.37	0.3	0.25	53
Tripolyphosphate	15	0.60	1.3	0.75	19
	45	0.98	1.2	0.72	33
	70	0.48	0.4	0.25	58
Imidodiphosphate	15	0.70	1.3	0.75	19
	45	1.00	1.0	0.65	30
	70	0.32	0.3	0.20	54
Pyrophosphate	15	0.60	1.3	0.75	18
	55	0.96	1.0	0.65	35
	80	0.45	0.3	0.25	60

The parameters defining the transformation (ΔA_{633} , ΔCa^{++} , ΔOH) were measured as described in the text.

Table 5.2 Effect of increasing concentration of Type II inhibitors on I_t .

INHIBITOR	CONCENTRATION (μ M)						
	15	30	50	100	150	200	300
ATP	20	22	35	> 1h	>1h	> 1h	> 1h
ADP	18	20	30	>48	>1h	> 1h	> 1h
Phosphonoformate	17	20	28	45	>1h	> 1h	> 1h
2-phosphoglycerate	17	18	22	30	41	52	> 1h
3-phosphoglycerate	17	17	18	21	27	33	49
Mg ²⁺	17	17	18	21	25	29	36
Citrate	17	17	18	19	21	23	27

Data shown represent time of induction (I_t) at selected concentrations and, unless otherwise shown, the time given as expressed in minutes.

The parameters defining the transition (ΔA_{633} , ΔCa^{++} , ΔOH) did not change from the control values listed in Table 5.1.

>1h indicates no transformation was detected over the time period monitored.

group was compared. Samples were taken for TEM analysis before, during and after the transformation, and Plate 5.1 shows the typical appearance of spherical particles before the transition and of needle-like particles after the transition.

The initial spontaneous precipitation of ACP due to the addition of base appeared unaltered by the presence of inhibitors, an observation also supported by light scattering and calcium-ion activity measurements. Subsequently, as described by Eanes et al. (1976), the spheres increased in diameter, although addition of an inhibitor caused a decreased rate of growth of the ACP spheres. Just prior to the transformation all particles were of similar appearance, but during the transition a change in morphology becomes evident with the spheres of ACP appearing indistinct and needle-like deposits appearing - again a phenomenon previously noted (Eanes et al., 1976). After the transition, needles of HAP usually appear except when high concentrations of Type I inhibitors are present. Furthermore, the turbidity of the suspensions then remained essentially constant over the time monitored (30-60 min) suggesting the time course of the Ostwald ripening process (Despotovic et al., 1975; Nancollas and Mohan, 1970) was too long for detection in these experiments.

As shown in Chapter 4, synergism between Mg^{++} , citrate and PC exists for the inhibition of HAP formation. Whereas the interactions between Mg^{++} and citrate, and PC and Mg^{++} , could be rationalised as due to chelation, it was noted that the interaction between PC and citrate could not

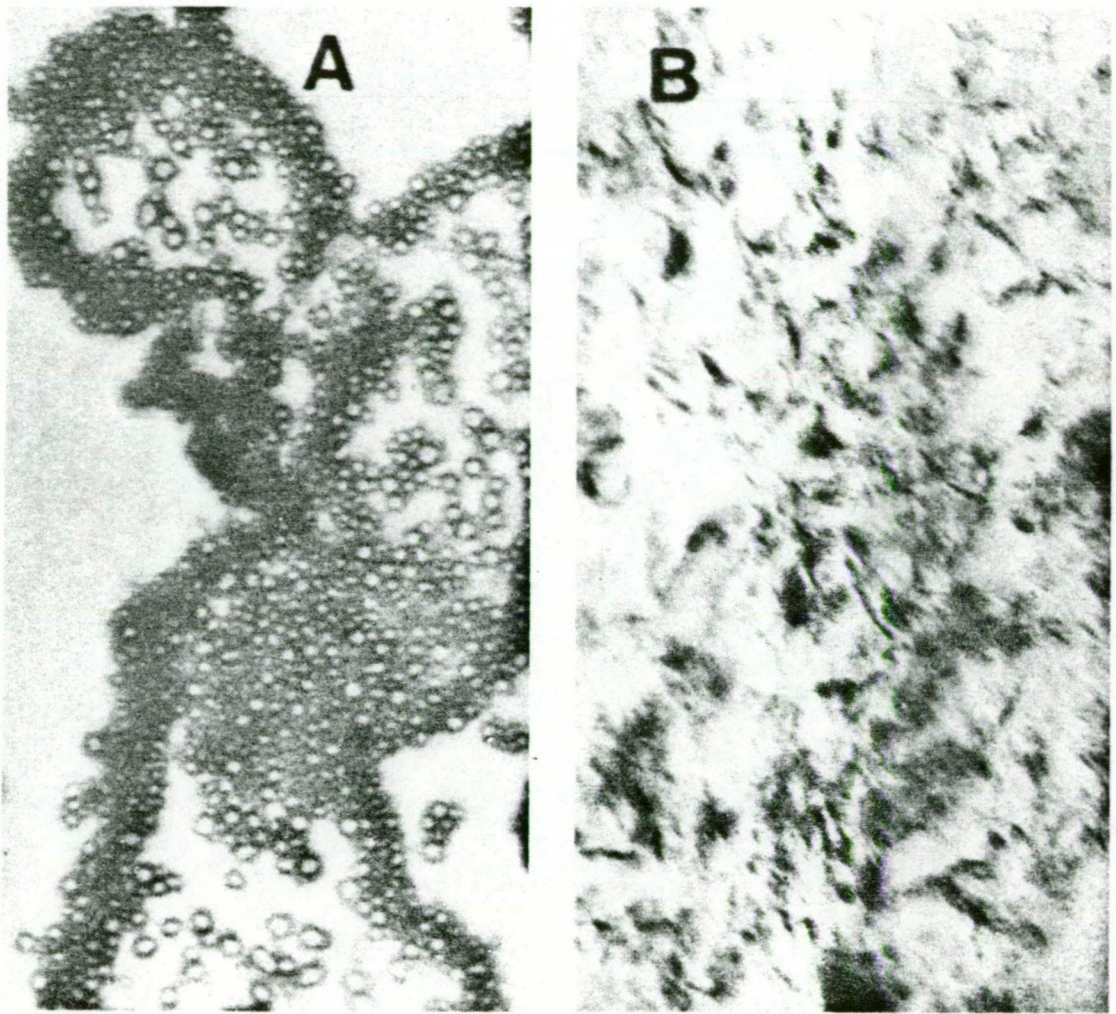


Plate 5.1 Typical transmission electron micrographs (TEMs) of calcium phosphate deposits.

A : before conversion (x200,000)

B : after conversion (x400,000)

be so well understood. However, the results in this chapter indicate that PC and citrate are of different inhibitor types (as defined here) and act by different mechanisms. This partially substantiates the claim made in the preceding chapter that they bind to different sites to efficiently block active growth sites. To examine whether this observation was an example of a general phenomenon, pairs of inhibitors (either from the same group or one from each) were tested and the results are given in Table 5.3. When pairs of inhibitors were from the same group no synergism was observable: the effect on I_t was purely additive. Notably however, when one inhibitor was Type I and the other Type II, synergism was apparent.

5.4 DISCUSSION

The finding that PC (a polycarboxylic monophosphate) inhibits HAP formation requires a re-appraisal of the suggestion that at least two ester phosphates need to be present. The observation that phosphonoformate and phosphonoacetate - carboxylic monophosphates - potently inhibit, whereas carboxylic acids do not, suggest that for potent inhibition the minimum structural requirement is a phosphate group and an acidic group, either a carboxylic or preferably an additional phosphate group. This latter consideration arises from the fact that, for example, pyrophosphate is a more potent inhibitor than phosphonoformate.

There are situations nevertheless, where this rule needs qualification: polycarboxylic, phosphate-free molecules will inhibit (Hay et al., 1979), but the minimum number

Table 5.3 Effect of compounds on the inhibition of hydroxyapatite formation when added in pairs.

COMBINATION*	INHIBITOR TYPE	ΔI_t (min) [†]	ΔI_t (exp.) [‡]	DIFFERENCE (min) [$\Delta I_t - \Delta I_t$ (exp.)]	SYNERGISM
PC	I	20	—	—	—
PNP	I	33	—	—	—
3-PG	II	9	—	—	—
Cit.	II	32	—	—	—
PA	II	16	—	—	—
PC + 3-PG	I + II	41	29	+12	YES
PC + PNP	I + I	54	54	0	NO
Cit. + PNP	II + I	100	49	+51	YES
PA + PNP	II + I	81	64	+17	YES
PA + Cit.	II + II	48	48	0	NO
3-PG + Cit.	II + II	28	26	+2 ^o	NO
PA + 3-PG	II + II	41	40	+1 ^o	NO

Abbreviations: PC = phosphocitrate, PNP = imidodiphosphate, 3-PG = 3-phosphoglycerate, cit. = citrate, PA = phosphonoacetate

* Amounts used were: 3.3mg PC, 12mg PNP, 16mg 3-PG, 76mg cit., 20mg PA

[†] I_t s were determined at pH 7.4, 20°C, as described in the text.

[‡] ΔI_t (expected) was calculated on the basis of an additive effect of inhibitors on HAP formation.

^oDifferences are not significant.

of carboxylic groups required for inhibition is as yet unknown. Formic and oxalic acids do not inhibit, but citric acid is a very weak inhibitor. Notably, the dehydroxyl and dehydro derivatives of citric acid, namely tricarballic acid and aconitic acid, fail to inhibit (Tew et al., 1980) so the hydroxyl group, perhaps due to its position in a tetrahedral environment, must be associated with the weak inhibitory ability of citrate. Certainly it has been implicated in the Ca^{++} -binding ability of citrate (Kieboom et al., 1978). However, as indicated by the ability of phosphonoacetate and phosphonoformate to potentially inhibit HAP formation, hydroxyl groups in compounds with phosphate groups are not needed for potent inhibition. Further, 3-phosphoglycerate inhibits, but in contrast o-phosphoserine, in which the hydroxy group is replaced by an amino group, does not inhibit over the concentration range studied. Thus, although amino acids bind to HAP (Kresak, 1977) the amino group must alter the binding characteristics sufficiently to abolish inhibition.

The types of functional groups are of primary importance in determining whether a compound inhibits or not, but secondary factors, including (i) the number of active groups, (ii) their proximity, (iii) stereochemical arrangement, (iv) steric factors, (v) lipophilicity, and (vi) lability of the molecule, also influence inhibitor potency.

In general, as the number of anionic groups increases so does inhibitor power. Thus ATP is more powerful than ADP, tripolyphosphate is better than PP_i , and PC is stronger than 2- and 3-phosphoglycerate. As mentioned above, sufficient carboxylic groups will confer inhibitory activity on a molecule but, as with all inhibitors, the potency of

inhibition is modified by the proximity of the active groups and their stereochemistry.

The proximity of the groups is vital since the closer the anionic groups the more strongly a molecule inhibits HAP formation. Thus phosphonoformate is more potent than 2-phosphoglycerate which is more powerful than 3-phosphoglycerate. With macromolecular inhibitors this also holds since polyaspartate is more potent than polyglutamate of similar molecular weight (Hay et al., 1979). Further, it follows that a linear arrangement of active groups should be less potent than an irregular arrangement which allows for a closer 'packing' of active groups. This may explain in part why PC is so much more potent than tripolyphosphate.

The results presented here also permit another important stereochemical factor to be elaborated: the hybridization of the atom 'radiating' chelating side arms. This appears to be important since it has been noted that EHDP is a disproportionately stronger inhibitor than nitrilotri(methylene phosphonic acid) when assessed on the number of anionic groups present (Meyer and Nancollas, 1973). Thus the stereochemical arrangement of chelating groups about the tetrahedral ' sp^3 ' carbon is more suitable to binding to active sites than about a trigonal nitrogen. Again, such stereochemical considerations may be another reason phosphocitrate is such a potent inhibitor. If the anionic groups are bound to an ' sp^2 ' hybridized carbon (i.e. double bonded carbon), the molecule no longer inhibits as indicated by the fact that the inhibition is seen with phosphonoacetate but not with phosphoenol pyruvate. This may reflect that

the altered 'sp²' bond angle splays out the chelating groups so that they no longer bind sufficiently to the active sites.

The effect of substitution of a sterically bulky group onto a parent inhibitor is demonstrated by PP_i and ADP. The inhibitory capability of ADP in comparison is decreased, perhaps reflecting decreased ability to fully bind the active sites. This phenomenon has also been observed by Hay et al. (1979); digestion of macromolecular inhibitors yielded fragments showing more potent crystal-growth inhibition. In general then, molecular size opposes the effects of increasing the number of groups and proximity by rendering binding to active sites less efficient.

Compounds tested in this study were hydrophilic but it should be noted that certain anionic molecules may disrupt HAP formation by a unique mechanism. Wuthier and Eanes (1975) have shown that anionic phospholipids, phosphatidyl serine for example, will stabilise ACP if added after initial precipitation. However, if the amorphous precursor is formed in the presence of phosphatidyl serine then its inhibitory effect is not as pronounced. It was suggested that this was due to phosphatidyl serine altering the structure of the amorphous phase by micelle formation, such that its surface area was increased, aiding the surface-dependent processes involved in HAP formation. According to the criteria outlined here, phosphatidyl serine should not be an inhibitor, and indeed the concentrations required by Wuthier and Eanes (1975) to produce inhibition are too high for it to be considered as a potent inhibitor.

From the data presented here it is evident that as

inhibitors EHDP > imidodiphosphate > PP_i . The order may be partly explained by increasing lability on calcium phosphate surfaces which are known to catalyse transphosphorylation reactions (Francis, 1969; Krane and Glimcher, 1962; Fleisch et al., 1968). Blumenthal et al. (1977) have proposed that ATP inhibits HAP formation until a sufficient amount is removed by hydrolysis, whereas Hay et al. (1979) have suggested that for stable peptide inhibitors precipitation occurs when the inhibitor concentration has been reduced by interaction with newly formed nuclei. For the phosphoric ester compounds studied in this work, it is probably a combination of these processes that eventually permits transformation of ACP into HAP.

All anionic inhibitory compounds are Ca^{++} chelators, but inhibition is not due to chelation in itself since EGTA does not significantly inhibit HAP formation. Neither is activity due to the attachment of a sterically bulky group to a phosphate moiety since *p*-nitrophenyl phosphate (with a P-O-C linkage) and 1- and 2-aminoethyl phosphonic acid (with a P-C linkage) failed to inhibit the transformation. Inhibition might have been expected if it was difficult to incorporate sterically bulky molecules into the HAP lattice, thus making HAP formation energetically less favoured.

What appears obligatory for inhibition to occur is adsorption onto calcium phosphate surfaces. This is a well-known process, HAP adsorbing, for example, nucleotides (Kawasaki, 1978), pyrophosphate (Fleisch et al., 1968), phosphonates (Francis, 1969), and, as shown here, PC and citrate. ACP also adsorbs compounds of similar types (Termine

and Conn, 1976). By measuring the surface area of HAP, Meyer and Nancollas (1973) showed that inhibitors do not cover the entire surface, leading to the proposal that inhibitors act by binding to active growth sites which are defect sites in the crystal lattice, a phenomenon general to all crystal types. The results presented here concur with the proposal of adsorption being a universal feature of inhibition, but turbidimetric, base titrimetric and calcium-ion activity measurements have allowed recognition of two inhibitor types (see Figure 5.5 and 5.6).

TEM evidence indicates that prior to transformation ACP spherules in all cases are of similar appearance. After the transformation, however, aggregates of needle-like deposits are visible, although dimensions of the individual particles are too small for comparative measurements. Since the amount of post-translational solid found in the presence of Type I inhibitors at the maximum change in turbidity, as indicated by calcium and base titrimetric measurements, is marginally less than with the control or with Type II inhibitors (see Figure 5.6), the magnitude of the turbidimetric changes is interpreted as due to the formation of many, very small particles during the conversion process. In contrast, Type II inhibitors do not alter the dimensions of the particles involved in the transition compared to the control, even though the onset of the conversion process is delayed, sometimes as long as with Type I inhibitors. Hence the grouping does not just reflect the inhibitor potency.

Needles of HAP are usually formed after the conversion process; this does not happen when a sufficiently high

concentration of Type I inhibitor is present. No transformation occurs and ACP spherules are indefinitely stabilised. With concentrations of Type I inhibitors a little less than that shown to be required to completely block conversion, it is still possible to detect a small degree of transformation - for example 10% of the control value at 60 μ M EHDP (see Figure 5.6) - but not enough for TEM to reveal HAP needles. Although no direct evidence is presented here, it might be predicted that at some concentrations of Type I inhibitors, when ACP is only partly converted to HAP, ACP spherules co-exist with HAP needles after the incomplete transformation.

Whatever the morphological subtleties that are responsible for the nature of the turbidity changes, the fact remains that turbidimetric data (ΔA_{633}) clearly group inhibitors into two classes. It is suggested that the phenomenon underlying this observation is that Type I inhibitors are crystal growth inhibitors and in fact most of them are established as such. Potent inhibition is not due to calcium chelation itself but is considered to be the result of adsorption onto ACP and HAP (Meyer and Nancollas, 1973; Fleisch et al., 1968). Type I inhibitors then, in a rationale based on the work of Posner and his co-workers (Posner and Betts, 1975; Posner et al., 1978), stabilize HAP embryos and inhibit subsequent growth by binding to active sites and poisoning heterogeneous nucleation. At high concentration of inhibitors, embryos fail to reach their critical size and re-dissolve which explains the decreasing turbidity change. The concomitant fall in base consumption and calcium-ion depletion confirms the decreasing extent of conversion of ACP to HAP occurring at these concent-

rations as do TEM data. Type II inhibitors, in comparison, are considered to act mainly by decreasing the lability of ACP. Both classes bind to ACP and HAP (Termine and Conn, 1976; Tew et al., 1980) and it therefore appears that Type I (crystal-growth) inhibitors either bind with higher affinity to the defect sites on HAP from which crystal growth proceeds, or that these sites are different to those bound by Type II inhibitors, or both. There is ample evidence for both processes. For example, nucleotides elute off HAP chromatography columns in an order reflecting binding affinity (Kawasaki, 1978). As to the nature of the binding sites on HAP, there is evidence indicating multiple binding sites exist even for closely related inhibitors. For example, Jung et al. (1973) have demonstrated that there are two classes of binding sites for PP_i , EHDP and Cl_2MDP and furthermore, that at high concentrations of PP_i , HAP binds more inhibitor molecules than can be predicted by the Taves and Reedy (1969) model.

Although inhibitors have been grouped into two types based on the manner by which their increasing concentration affects the parameters defining the transition, it does not necessarily indicate that only two mechanisms underlie this division. With Type I (crystal-growth) inhibitors it is likely that binding to active growth sites on HAP is the fundamental mechanism, although, as has been mentioned, it is possible that even closely related compounds, such as PP_i and the diphosphonates, may bind to different sites on HAP and with varying affinities. With Type II inhibitors ACP is considered to be the main locus of action; but diverse

mechanisms must be responsible for their inhibitory action since, for example, Mg^{++} and citrate³⁻ will bind to different sites.

Further, it has been suggested that ATP prevents HAP embryo formation until it is removed by surface-catalysed hydrolysis (Blumenthal et al., 1977); Type II inhibitors such as citrate and phosphonoformate, which are not hydrolysed, cannot inhibit by this mechanism.

The recognition that inhibitors may or may not affect the extent of conversion is not new. Blumenthal et al. (1975) found that HAP formed in the presence of ATP was crystalline; with EHDP present HAP was only partly crystalline. This is consistent with the rationale presented here. The suggestion that some inhibitors do not alter the dimensions of the particles involved, again, has been observed previously. Boskey and Posner (1974) demonstrated that Mg^{++} , a Type II inhibitor from our data, did not alter the dimension of the crystallites formed relative to the control, and suggested that this was due to its locus of action being ACP. This is exactly what is proposed here for all Type II inhibitors: the apparent primary site of action is at the ACP surface. Type I inhibitors also possess the capacity to decrease ACP lability, but it is secondary to the crystal-growth poisoning ability.

The synergism demonstrated to occur when a Type I and Type II inhibitor are added together can be understood on the basis of binding to different active sites on ACP and HAP. When this occurs such an efficient coverage of the surfaces is achieved that growth and nucleation are more effectively hindered than in the presence of just Type I

or Type II inhibitors. It is in this context that the synergism between PC and citrate, described in the previous chapter, can be considered. The phenomenon of synergism emphasises that in physiological fluids complex interactions between ions may occur.

The data reported here then, allows for the prediction of compounds that will inhibit HAP formation, and offer a preliminary estimate of potency. An explanation has been given of the mechanisms at the calcium phosphate surfaces which may underlie the separation of inhibitors into two classes, but, as yet, no attempt has been made to elucidate any structural features of a molecule which will a priori indicate whether it is a Type I or Type II inhibitor. Indeed, no obvious pattern readily emerges, but it is tempting to ascribe Type I inhibition to the presence of one phosphate group and, in close proximity, either another phosphate or multiple carboxyl groups. However, nucleotides need special consideration because whereas tripolyphosphate and PP_i are Type I inhibitors, ATP and ADP are Type II inhibitors. PP_i is not completely hydrolysed before transformation (Fleisch et al., 1968), but it could be that nucleotides are hydrolysed before the conversion process can occur. Certainly this has been shown for ATP (Blumenthal et al., 1977) but evidence is lacking on the stability of ADP under these conditions. If ADP is not hydrolysed it may be that it binds poorly to those active sites on HAP that bind Type I inhibitors, perhaps due to the sterically bulky adenosine group. Kawasaki(1978) has shown that the adenosine moiety sterically obstructs one C site per molecule and in our rationale this

decrease in coverage may be sufficient to permit HAP crystal growth to proceed unhindered, at least over the concentration range studied here. Alternatively, it may be that this inability of ADP, and indeed ATP, to bind as efficiently as PP_i and tripolyphosphate to HAP embryos, makes them more susceptible to the surface-catalysed hydrolysis. Whatever the active site(s), the decrease in accessibility of ADP and ATP (relative to pyrophosphate and tripolyphosphate) for these binding sites is likely to explain the profound difference in inhibitory types. This accessibility consideration consequent upon the molecular weight, together with the nature of groups outlined above, gives some criteria for predicting which group an inhibitor may be placed.

The study described here has confined itself essentially to low molecular weight compounds that potently inhibit HAP formation, that is increase I_t at low concentrations (5-100 μ M) and these inhibitors appear to fall into two groups, Type I or Type II inhibitors. However, this finding does not preclude the possibility that there may be other types of inhibitors which affect the ACP to HAP transition in a different manner. For example, it has been suggested that bulky anionic macromolecules can bind to ACP and HAP sufficiently to sterically interfere with the conversion process (Hay et al., 1979; Blumenthal et al., 1979). This appears to result in proteoglycans not significantly affecting I_t but decreasing the rate and extent of the transformation (Blumenthal et al., 1979) at concentrations too high for these polysulphonated macromolecules to be considered potent inhibitors in vitro. This is consistent with the data presented

here on the structure-activity relationship of low molecular weight inhibitors but does indicate that macromolecules open up new mechanistic possibilities for inhibition.

The results presented in this chapter have led to the proposal of guidelines which allow prediction of the ability of a molecule to inhibit HAP formation. Secondary factors will influence the degree of potency exhibited and also determine if a molecule is an inhibitor of crystal-growth or not. This latter characteristic is the basic difference between the two groups or inhibitors that have been recognised in this study.

5.5 SUMMARY

1. The transition of ACP into HAP can be monitored by not only base titration but also Ca^{++} -ion activity in solution, and turbidity.

2. Inhibitors of the ACP-HAP transformation must possess at least one phosphate group and either a carboxylic moiety or preferably a phosphate group; otherwise they will not be potent inhibitors.

3. Secondary factors influence inhibitory ability: (i) the number of active groups, (ii) their proximity, (iii) stereochemical arrangement, (iv) steric factors, (v) lipophilicity, and (vi) lability of the molecule.

4. Low molecular weight inhibitors can be grouped into two classes depending on how they affect the parameters defining the transition of ACP into HAP: Type I (crystal growth) and Type II inhibitors.

5. Type I inhibitors are crystal-growth inhibitors and at high concentrations indefinitely stabilise the ACP spherules. PC, EHDP, tripolyphosphate, imidodiphosphate

and PP_i are Type I inhibitors.

6. Type II inhibitors act by a variety of mechanisms but do not alter the dimensions of the HAP crystallites formed after the conversion of ACP. Type II inhibitors are: ATP, ADP, phosphoformate, 2-phosphoglycerate, 3-phosphoglycerate, Mg^{++} and citrate.

7. A compound with a phosphate group and, in close proximity, either another phosphate group or multiple carboxyl groups will be a Type I inhibitor provided no bulky side-groups are present.

8. Pairs of Type I inhibitors, or pairs of Type II inhibitors have an additive effect on inhibition. Type I and Type II inhibitors added together have a synergistic effect.

CHAPTER 6

THE NATURAL OCCURRENCE OF PHOSPHOCITRATE

6.1 INTRODUCTION

The potent inhibitory activity of PC in vitro emphasises the need to prove whether or not PC occurs in biological tissues, and if it does, whether it could exert an influence on biological calcification systems. Although the properties of the molecule strongly support the contention that its physiological role is in controlling calcification, there is no conclusive evidence to date that PC has ever been isolated and confirmed as a natural biological inhibitor. Indeed Lehninger (1977), in his reported studies, suggested that the inhibition seen might not be due to PC but to some impurity isolated in the fraction supposedly containing PC. On the other hand, Tew et al. (1978) concluded that since a fraction isolated from mitochondria by ion-exchange chromatography inhibited the transformation of ACP into HAP, it therefore contained PC. However, the agent responsible for inhibition cannot be unambiguously identified in this manner, since many compounds inhibit the transformation process.

Howard (1976) made a tentative proposal that the biological inhibitor isolated by ion-exchange chromatography from urine and mitochondria was PC. However, the lability of the biological inhibitor recorded some years earlier by Howard et al. (1967) did not match the data published by Meyer et al. (1959) on the lability data for PC. Further, Howard presented evidence suggesting that the biological

inhibitor could be formed artificially on the ion-exchange column merely by eluting citrate and phosphate under acid conditions. Therefore the question of whether PC did indeed occur biologically, or was only an artefact of the chromatographic procedure, remained unanswered.

The problems encountered by these workers have now been overcome by the methodology developed here permitting isolation of material from biological sources and its subsequent comparison with synthetic PC. Further, although NMR spectral data together with mass spectroscopy provide information on the structural nature of a compound, unfortunately it is not always possible to obtain sufficient amounts from biological material for analysis by these methods. Consequently a more versatile approach has been adopted, whereby a range of techniques are used for characterising PC, more valuable than using any single technique. One other possible criticism has already been eliminated, namely the demonstration that chromatography at neutral pH avoids PC artefact formation. The data also indicate that acidification and neutralisation does not cause PC production, justifying the use of acid extraction techniques for tissue preparation.

This chapter, therefore, reports on the use of ion-exchange chromatography to isolate naturally-occurring PC from various sources and its subsequent detection and characterisation by a range of techniques, including enzymatic and chemical citrate assays, TLC, analytical isotachopheresis and high-voltage electrophoresis. Kidney, intestine and bone, which are the primary sites of Ca^{++} metabolism, were

investigated because PC can influence calcifying systems. Liver was also included for comparison. In addition, normal urine was also investigated to determine if PC was present, since under pathological conditions calcifications may form in the urinary tract.

6.2 METHODS

6.2.1 COLLECTION AND TREATMENT OF BIOLOGICAL SAMPLES

6.2.1.1 Whole tissue

The possibility that PC occurs in nature was initially examined by subjecting biological extracts to chromatography and analysing relevant fractions. The biological extracts were prepared from kidneys, liver or intestine excised from animals (rats or rabbits) killed by cervical dislocation. Metabolic transformation (if any) of PC was minimised by freeze-clamping the tissue samples on excision. The frozen tissue was then pulverised in a stainless steel pestle and mortar, and the powder added to ice-cold perchloric acid (10ml/g tissue) for protein denaturation.

6.2.1.2 Bone

Bone was collected essentially as described by Perkins and Walker (1958). Tibias and fibias were dissected free from adherent tissue before the bones were split and the marrow removed using a jet of water. The bones were stored in liquid nitrogen before being pulverised. The bone fragments were suspended in ice-cold 0.5M HClO_4 (5ml/g), Dowex 50W-X8 (1g/g bone) added and the mixture vigorously stirred for several hours. It was evident that not all of the bone fragments had been completely dissolved but a sample for

analytical purposes was obtained after filtering the mixture to separate the resin and bone particles from extracted compounds.

6.2.1.3 Urine

Mid-morning (9-11 a.m.) urine samples were collected from male medical students in two 10L flasks containing a few crystals of thymol each. Urine so collected was processed within 8h. Between 2 and 6L of urine was filtered through a Hollow Fibre Dialyser/Concentrator (Amicon Model DC2) giving a filtrate that was immediately loaded onto a column of AG 1-X8 (HCO_3^-).

6.2.1.4 Mitochondria

6.2.1.4.1 Kidney and liver

The procedure used to isolate mitochondria from kidneys and liver was essentially that of Bustamante *et al.* (1977). Accordingly, excised tissue was cleansed of connective tissue, blood and fat before mincing with scissors to fragments smaller than 0.5 x 0.5cm. A suspension of fragments in ice-cold 0.25M sucrose, 0.15mM EDTA and 0.01M Tris-HCl, pH 7.4 (10ml/g tissue) was homogenised using a chilled Potter-Elvehjem glass homogeniser and a motor-driven Teflon pestle. Three strokes of the pestle were sufficient to prepare the homogenate which was then layered over 0.34M sucrose (10ml/30ml of homogenate). The tubes were centrifuged at 800g for 10 min at 2°C before 0.25M sucrose layer was removed by pipette. The 0.25M sucrose suspension was then centrifuged at 10,000g for 15 min to give a pellet containing

mitochondria. The supernatant was poured off and the mitochondrial pellet resuspended in 0.25M sucrose before centrifugation for 15 min at 10,000g. The supernatant was again poured off, the pellet suspended in 0.25M sucrose and the volume of the mitochondrial suspension recorded. An aliquot (0.5ml) was taken for subsequent analysis. To the bulk suspension an equal volume of ice-cold 1.0M HClO_4 was added. This suspension was homogenised in a Potter-Elvehjem glass homogeniser by two strokes of the Teflon pestle before the protein and perchlorate were removed by centrifugation.

6.2.1.4.2 Intestine

Rat intestinal mitochondria were prepared as outlined by de Jong *et al.* (1978). The small intestine from the ligament of Treitz to within ca. 10cm of the ileocaecal sphincter was excised and immersed in ice-cold isolation medium comprising 0.154M NaCl, 10mM Tris-HCl and 5mM EDTA, pH 7.4. A glass canula was inserted in one end and held in place with cotton thread, whilst the luminal contents were flushed out using a 10ml syringe filled with the isolation medium. The mucosal surface was exposed by cutting open the intestine with a pair of blunt-nosed scissors and then the intestinal epithelium was scraped off using a microscope slide and transferred to ca. 20ml of homogenisation medium (0.25M sucrose, 5mM EDTA, pH 7.4 and 0.1M Tris-HCl). The suspension was poured into a stainless steel bomb (Yeda Press, Yeda Scientific Instruments, Rehovot, Israel) and equilibrated under a pressure of 150 lb/inch² of nitrogen for 30 min. The outlet valve was then opened to allow slow expulsion of the suspension and the volume was adjusted

to 40ml and 1 drop of anti-foam (Dow Corning Corp., Midland, U.S.A.) was added before the suspension was homogenised in a Potter-Elvehjem glass homogeniser with three strokes of a Teflon pestle. The mitochondria were then prepared as described above.

6.2.2 BIURET TEST FOR PROTEIN

The biuret test for protein (Gornall *et al.*, 1949) was used to quantitate the amounts of mitochondrial protein isolated from different sources. Biuret reagent (2ml) and 1N NaOH (1ml) was added to 100-200 μ l of the mitochondrial suspension. The mixture was stirred, then left for 15-30 min before reading at 540nm. Controls included mitochondria suspended in 3ml of NaOH and a solution of 1ml NaOH in 2ml biuret reagent. Standards of 1-10mg bovine serum albumin (Grade A) were routinely included.

6.2.3 DEPROTEINISATION

Whole tissue and/or mitochondria were deproteinised by either of two techniques.

6.2.3.1 HClO₄ deproteinisation

Whole tissues (10ml/g wet weight) or mitochondria (5ml/100mg) were homogenised for 30s in ice-cold 0.5M HClO₄ with a top drive blender (Silversen Top Drive, Waterside, Chesham, Buckinghamshire, England) before centrifugation at 8,000g for 10 min to remove denatured protein. The chilled supernatant was immediately neutralised with cold conc. KOH then kept on ice for 10-15 min before the white precipitate was removed by centrifugation. The supernatant contained the metabolites extracted from the biological samples.

6.2.3.2 Trichloroacetic acid (TCA) deproteinisation

The TCA extraction procedure was essentially that of Khym (1975). Either 2ml of 6% TCA was added dropwise to blood (1ml) or 1ml of 12% TCA was added dropwise to ca. 800mg of frozen tissue powder. The suspensions were vigorously stirred and then centrifuged at 2,000g for 5 min. The supernatant was removed and 4ml of a solution of tri-n-octylamine in Freon (8.8ml in 50ml) was added. The stoppered tubes were shaken horizontally for 15-20 min to ensure thorough extraction before the upper layer was removed, leaving a deproteinised aqueous layer (pH 6.0-7.0) which was lyophilised.

6.2.4 ION-EXCHANGE CHROMATOGRAPHY

The chromatographic procedure used to isolate PC comprised two steps: (i) batch-wise elution to produce a crude PC fraction free from citrate and P_i , and (ii) gradient elution to give a PC peak eluting off the column at a characteristic volume.

6.2.4.1 Batch-wise purification

6.2.4.1.1 Whole tissue

5-10 μ l of [14 C]-citrate was added to extracts from whole tissue (5-25g) and an aliquot (0.5ml) counted after the addition of scintillant [3g 2,5-diphenyloxazole (PPO) in ethanol, ethylene, glycol, xylene and triton X-100, 106:37:600:257, v/v]. The tissue extract was then loaded at room temperature onto a 3 x 10cm AG 1-X8 (HCO_3^-) column at a flow rate of 1-2ml/min. An aliquot (0.5ml) from the eluant was taken, scintillant added and the sample counted.

From information on the number of counts present in the sample before and after passage through the ion-exchange column, the degree of binding of citrate was calculated. With a 3 x 10cm column, >95% binding was achieved.

The column was eluted with 1-1.5 L of 0.27M NaHCO_3 before 1L of 0.45M NaHCO_3 was passed through the column. This latter eluant was collected under refrigeration before decarbonation with AG 50W-X8. Sufficient AG 50W-X8 was added to the sodium bicarbonate eluant to alter the pH to 4-6. The mixture was filtered, the filtrate neutralised and lyophilised before being dissolved in 50-100ml of water. At this stage the solution was ready for the second chromatographic procedure.

6.2.4.1.2 Mitochondria and bone samples

Neutral extracts were loaded onto 1 x 10cm columns of AG 1-X8 (HCO_3^-) after the addition of 5-10 μl of [^{14}C]-citrate. Aliquots (0.5ml) of the extracts were taken before and after passage through the column enabling the binding of citrate to be estimated. Under these conditions, >95% binding was achieved and the column was eluted with 500-600ml of NaHCO_3 buffer which was collected in a chilled container. This fraction was decarbonated and subsequently treated to give a neutral crude PC fraction, as described above.

6.2.4.1.3 Urine samples

The high salt content of urine required the use of a large amount of resin in an attempt to fully find anions present. Accordingly, 2L of urine was passed through

a 5 x 50cm column of AG 1-X8 (HCO_3^-). Samples (0.5ml) of urine were taken before and after passage through the ion-exchange column and analysed for phosphate. Binding was >70% under these conditions. The column was eluted with 2L of 0.27M NaHCO_3 before 1.5L of 0.45M buffer was used to batch-elute PC. This fraction was collected in a chilled container, decarbonated and lyophilised as described above.

6.2.4.2 Gradient elution of PC

Lyophilised samples from the treated 0.45M fractions of the batch-wise eluted columns were taken up in 50-100ml of water and 5 μl of [^{14}C]-citrate added. These solutions were each loaded onto a 1 x 10cm column of AG 1-X8 (HCO_3^-) and the radioactivity present before and after passage through the column determined. In all cases >95% binding was obtained. The columns were eluted either individually or simultaneously as follows:

6.2.4.2.1 Treatment of individual columns

Five hundred ml of 0.27M NaHCO_3 was passed through the column to remove any traces of citrate before a linear gradient from 0.27 to 0.5M NaHCO_3 was applied to elute PC. Five hundred ml each of 0.27M and 0.5M NaHCO_3 was used to provide the linear gradient and 300 x 4ml fractions were collected and subsequently analysed.

6.2.4.2.2 Simultaneous elution of columns

When columns were treated simultaneously, one 1 x 10cm column of AG 1-X8 (HCO_3^-) was loaded with 50-100 μg of PC for comparative purposes. The columns were

connected to a Durrum 12 channel peristaltic pump (Model 11000, Palo Alto, California, U.S.A.) and the flow rate carefully equalised to 1ml/min. Then 500ml of 0.27M NaHCO_3 was passed through each column before applying a linear gradient to elute PC. For 'n' columns, 'n' x 500ml of both 0.27M and 0.5M NaHCO_3 were used to provide the gradient. One hundred and fifty ml of the gradient was collected from each column before 20 x 15ml fractions were simultaneously collected from each column for subsequent analysis.

6.2.5 CITRATE ANALYSES

Acidic conditions for the hydrolysis of PC prior to citrate analysis were achieved by adding 0.8ml of 5N H_2SO_4 into every fifth tube. Tube contents were then heated at 100°C for 4h before chemical citrate analysis as described in Section 3.2.1.1.1 using either 4,4'-methylene-dianiline or benzidine as the chromophore.

In some instances the fluorimetric coupled enzyme test for PC was used (Section 3.2.1.1.2).

6.2.6 SEPARATION TECHNIQUES

Citrate analyses indicated where PC eluted, so relevant tubes were pooled, decarbonated and lyophilised to give samples that were further analysed by TLC (Section 3.2.3.2), high-voltage electrophoresis (Section 3.2.3.3) and analytical isotachophoresis (Section 3.2.3.4). Where necessary the biological material was stained for either P_i or citrate (Section 2.3.3).

6.2.7 NMR SPECTROSCOPIC EXAMINATION

Rat kidneys were extracted with perchloric acid to give neutral extracts that were analysed by ^{31}P NMR spectroscopy (courtesy of Dr. Seeley, Oxford University). An external standard of PC in 200mM NaCl and at an identical pH to the sample was used for comparative purposes.

6.3 RESULTS

6.3.1 RECOVERY OF PC FROM HClO_4 AND TCA EXTRACTS

When [^{32}P]-PC was added to 1g of tissue, homogenised and then deproteinised using either HClO_4 or TCA (Section 6.2.1), a quantitative recovery of > 98% was obtained for each technique in at least 3 separate trials. Acidification and neutralisation of a solution of citrate and phosphate was shown, in Chapter 3, not to cause artefactual PC production. This observation, together with the fact that the HClO_4 extraction technique is more readily applicable to a larger scale operation, led to the use of this method as a routine.

6.3.2 ION-EXCHANGE CHROMATOGRAPHY OF BIOLOGICAL EXTRACTS

Ion-exchange chromatography was used to isolate PC from biological extracts. Some of these purified fractions were subsequently analysed by TLC, electrophoresis, isotachopheresis and enzymatic assay in order to confirm the identity of the compound eluting from the column.

6.3.2.1 Whole tissue

Extracts of rat and rabbit liver and kidneys when subjected to ion-exchange chromatography with linear gradient

elution, always gave a peak that eluted at a volume characteristic of PC and which contained citrate after hydrolysis. Figure 6.1 gives typical results obtained with whole tissues. Liver extracts from two rats, or kidneys from six rats were used, the data being confirmed on at least four separate occasions. For rabbit extracts, liver and kidneys were obtained on separate occasions from four different animals, with consistent results. Significantly, in all cases a compound which contained hydrolysable citrate eluted from the ion-exchange column at a volume characteristic of PC. In fact, when [^{32}P]-PC was added to one rat kidney extract the radioactivity eluted at exactly the same position as the peak containing the hydrolysable citrate. Further, quantitative recovery of [^{32}P]-PC was obtained.

6.3.2.2 Mitochondria

When mitochondria from rat or rabbit liver or kidney were isolated and fractionated, a peak containing hydrolysable citrate was observed and again this peak corresponded to PC, eluting at a characteristic volume. Typical results are shown in Figure 6.2 and each experiment was confirmed three times.

In a single experiment mitochondria from the intestine of three rats was subjected to ion-exchange purification and, once more, a peak eluting at the characteristic volume of PC and containing hydrolysable citrate was observed (Figure 6.2). Furthermore, [^{32}P]-PC eluted with this peak when added to a rat kidney mitochondrial preparation.

From the area under the curve for hydrolysable citrate,

Figure 6.1 Resolution of PC from kidney and liver extracts of rats and rabbits by ion exchange chromatography.

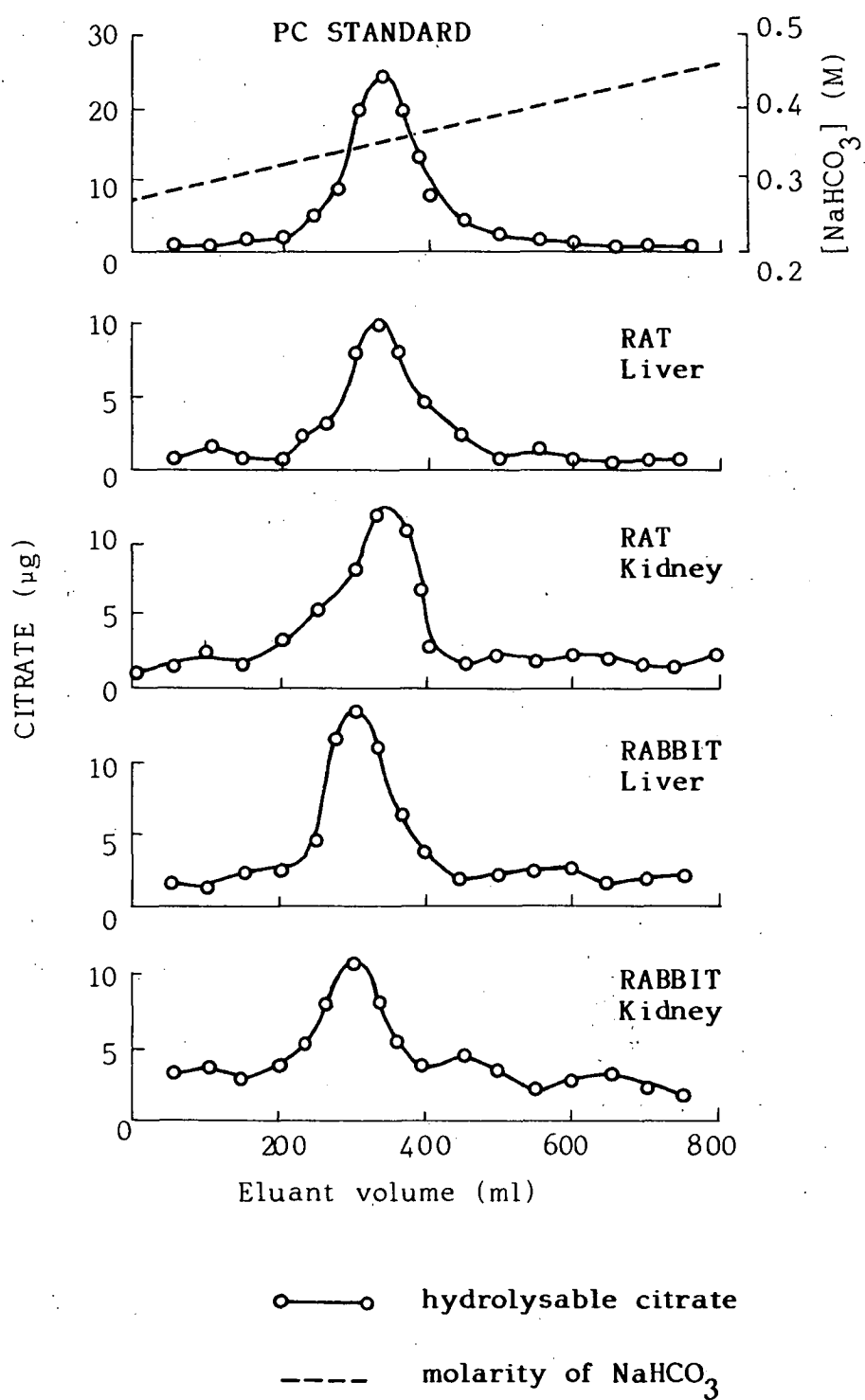
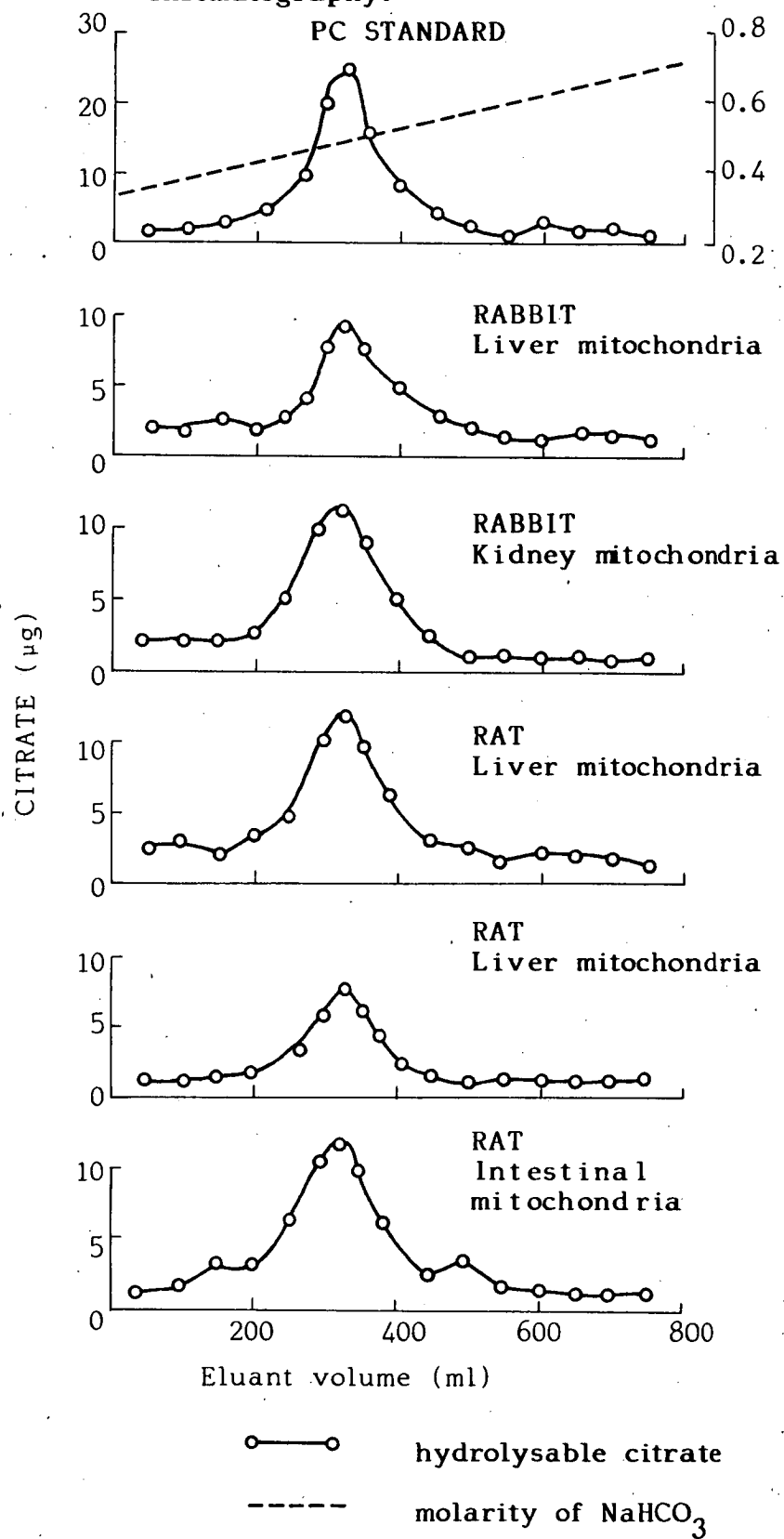


Figure 6.2 Separation of PC from extracts of rat and rabbit soft-tissue mitochondria by ion-exchange chromatography.



an indication of the initial amount of PC present in the biological extract may be calculated since mitochondrial protein was routinely analysed. A quantitative comparison (expressed as nmol PC/mg mitochondrial protein) is given in Table 6.1, indicating that kidney mitochondria from either species contained more PC than liver mitochondria. The richest source was rat intestinal mitochondria. It should be emphasised that in some experiments PC was not detected, perhaps due to some biological variation as yet undefined. Therefore these results can only be preliminary estimates of PC levels in mitochondria.

6.3.2.3 Bone

Treated bone samples from rabbit hind-legs were subject to ion-exchange chromatographic techniques and a qualitative indication of the presence of PC was obtained after citrate analysis. Although the amount of hydrolysable citrate detected in eluted fractions was very small, the consistent appearance of material in the eluant corresponding to the volume at which PC eluted nevertheless suggested that PC was indeed present (Figure 6.3).

6.3.2.4 Urine

Quantitation with human urine was not possible since the high salt content of urine prevented full binding to ion-exchange columns. Nevertheless, chemical citrate analysis indicated hydrolysable citrate was present, eluting at the same volume as PC. As with the analysis of bone, the amount of citrate present in the eluant was very small, but detected

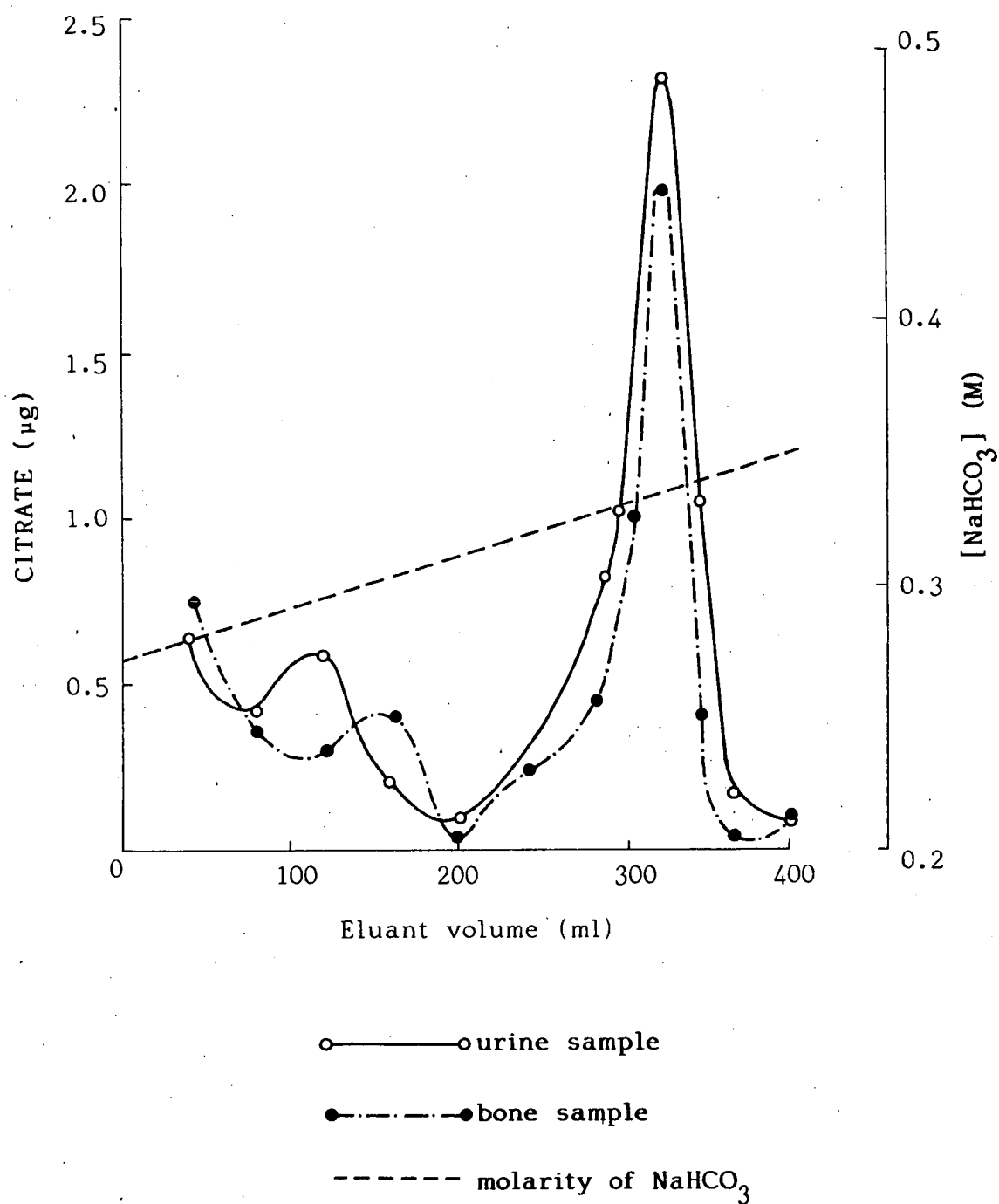
Table 6.1 Preliminary estimation of the amount of
PC present in mitochondria from various
sources.

TYPE OF MITOCHONDRIA	[PC] ($\mu\text{g}/\text{mg}$ mito.protein)
rat, rabbit liver	$0.4^{+0.2*}$
rat, rabbit kidney	$0.8^{+0.2*}$
rat intestine	1.2^{+}

*Data from at least 3 trials; mean ± 1 S.D.

⁺Data from 1 trial

Figure 6.3 Ion-exchange chromatographic separation of PC from bone and urine samples.



consistently over a range of tubes corresponding to the position PC is expected to elute off the column (Figure 6.3).

6.3.3 FURTHER ANALYSIS OF ISOLATED FRACTIONS

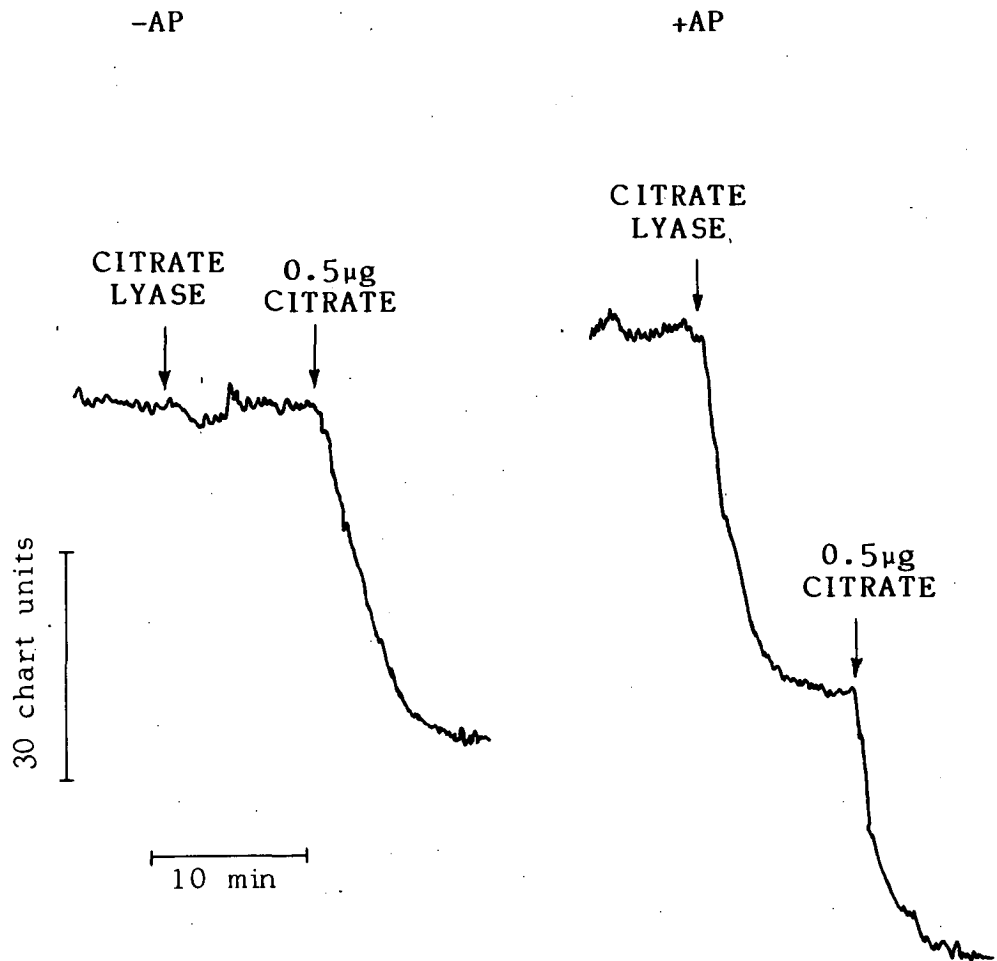
Having detected PC in the eluant of chromatographic columns, fractions containing PC from whole tissue and mitochondrial extracts were pooled, decarbonated and lyophilised so that further characterisation could be attempted. All the analytical procedures were used on rabbit liver and rat kidney mitochondrial extracts in order to ensure that the identity of the peaks eluting off the columns were PC. Once this was assured other samples were not subjected to the full range of analytical techniques.

6.3.3.1 Enzymatic analysis

Enzymatic analysis, which can determine both free citrate and PC, was used to examine rabbit liver and rat kidney extracts and all the mitochondrial extracts. Initial experiments involving the addition of PC into the biological fractions indicated that an incubation with alkaline phosphatase for 10 min at 37°C and pH 10 was insufficient to completely hydrolyse PC. However, it was established that PC was hydrolysed completely after 1h under these conditions.

Unhydrolysed and hydrolysed samples from each sample were analysed for citrate fluorimetrically (Section 3.2.1.1.2). After the reaction was complete 1.0 µg of citrate was added as an internal standard to check the kinetics of citrate lyase and to enable calculation of the amount of hydrolysable citrate present in the biological sample. Typically no free citrate (<1% total citrate) was detectable (see Figure 6.4).

Figure 6.4 Fluorimetric determination of phosphocitrate in a rat kidney extract using a coupled enzyme assay.



PC in the biological sample was analysed for citrate after hydrolysis of 100 μ of alkaline phosphatase at 37 C for 10 min.

\pm AP presence or absence of alkaline phosphatase (AP)

↓ point of addition of reagent

Since phosphatase digestion is necessary before citrate can be detected, then clearly the compound must be a conjugate of citrate and phosphate. Phosphocitrate is the only realistic candidate since citryl phosphates (phosphate esterified to carboxyl groups) are too unstable to occur naturally (Section 2.5).

The values for citrate were identical for the enzymatic and the chemical methods of assay; this constitutes an important validation for the estimates of mitochondrial PC concentration.

6.3.3.2 Separation techniques

Although the elution characteristics from ion-exchange columns and chemical and especially enzymatic analyses indicate that PC was present in biological samples, further proof was sought by comparing the material with synthetic PC using thin-layer chromatography, high-voltage electrophoresis and analytical isotachophoresis.

6.3.3.2.1 Thin-layer chromatography (TLC)

Biological samples applied to cellulose coated plates developed in an isobutyric acid based solvent (Section 3.2.2.2) gave a compound running at the same R_F to PC and which stained for phosphate after either chemical or enzymatic hydrolysis. This spot had an R_F quite different to that for either citrate or P_i . However, insufficient material was present for a citrate stain, to be successful so an alternative approach was adopted to investigate whether the material running with the same R_F as PC did indeed contain citrate.

Accordingly, a PC standard and biological sample were applied separately and subjected to the chromatographic procedure. The plate was marked into 2 x 2cm strips and bands of cellulose were scraped off the plate and material recovered by treating the cellulose with three lots of 4ml of MeOH/H₂O/1N H₂SO₄ (10:10:2). Cellulose was vigorously shaken with the eluting solvent then centrifuged. The supernatants for each band were pooled and lyophilised before hydrolysis at 100°C for 4h in 5ml H₂O. The samples were then analysed chemically for citrate and the results are given in Figure 6.5. Notably hydrolysable citrate was detected at the same R_F as authentic PC. In this system citrate runs 6-7cm from the origin and no free citrate was detected. Thus TLC indicated that the peak eluting from ion-exchange columns containing hydrolysable citrate was a phosphate and citrate containing compound with an R_F identical to PC.

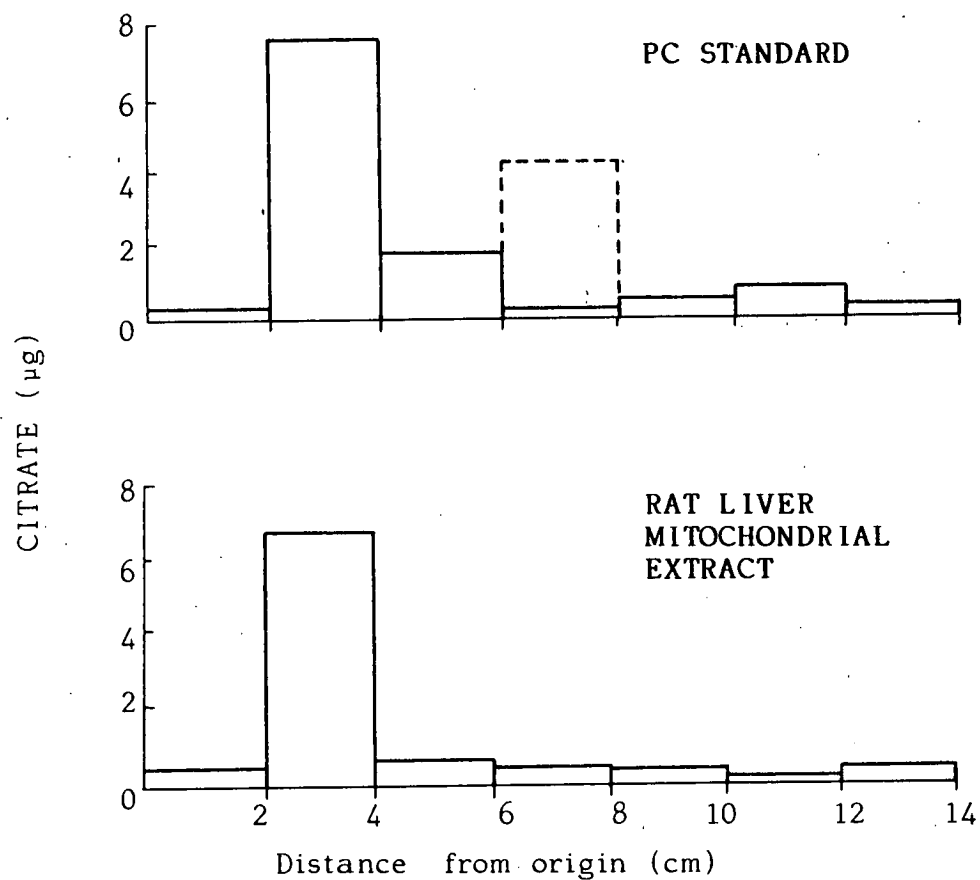
6.3.3.2.2 High-voltage electrophoresis

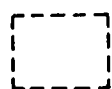
High-voltage electrophoresis indicated that a compound staining for phosphate and with the same mobility as PC was present in the biological samples. Again, insufficient material was present to allow specific citrate stains to be used. In contrast to TLC, citrate and PC are not resolved by electrophoresis, so the paper was not sectioned for subsequent elution and citrate analysis since this could not be a definitive test for PC.


6.3.3.2.3 Analytical isotachophoresis

Extracts from rabbit liver and rat kidney mitochondria were subjected to isotachophoresis with a view

Figure 6.5 Presence of PC in a rat liver mitochondrial extract determined by TLC.



 R_F of citric acid

 total citrate per fraction

to reinforcing the identification of the compound eluting from the ion-exchange column as PC. The conductimetric trace obtained after isotachophoresis is given in Figure 6.6. A component with a step height characteristic of PC was observable and when an internal PC standard was included it migrated with this zone.

In an extension of this approach the biological samples were incubated with alkaline phosphatase at a pH of 7. Incubating at a pH of 7 rather than 10 was necessary since adjusting the pH from 10 to 7 increased the salt content of the sample enough to interfere with subsequent analysis. Analytical isotachophoresis indicated that the PC zone had indeed decreased in length but had not been entirely eliminated because the pH is not optimal for complete hydrolysis. Nevertheless, the citrate zone in contrast had increased in width at the expense of PC and this behaviour is consistent with the component being PC.

6.3.3.3 ^{31}P NMR Spectroscopy

Figures 6.7a and 6.8a give the ^{31}P NMR spectra of rat kidney and liver perchloric acid extracts respectively. An external standard of PC (2 μmol) at the same pH as the biological samples was routinely included (Figures 6.7b and 6.8b). Peaks in the spectra are tentatively identified based on the work of Ogawa *et al.* (1978). The presence of a peak due to PC is difficult to detect due to proximity of resonances resulting from the phospholipids phosphatidyl choline (GPC) and phosphatidyl ethanolamine (GPE). Thus, unless PC is purified and concentrated before ^{31}P NMR spectroscopy, its

Figure 6.6 Isotachophoretic identification of PC from biological sources: detection of citrate after incubation with alkaline phosphatase

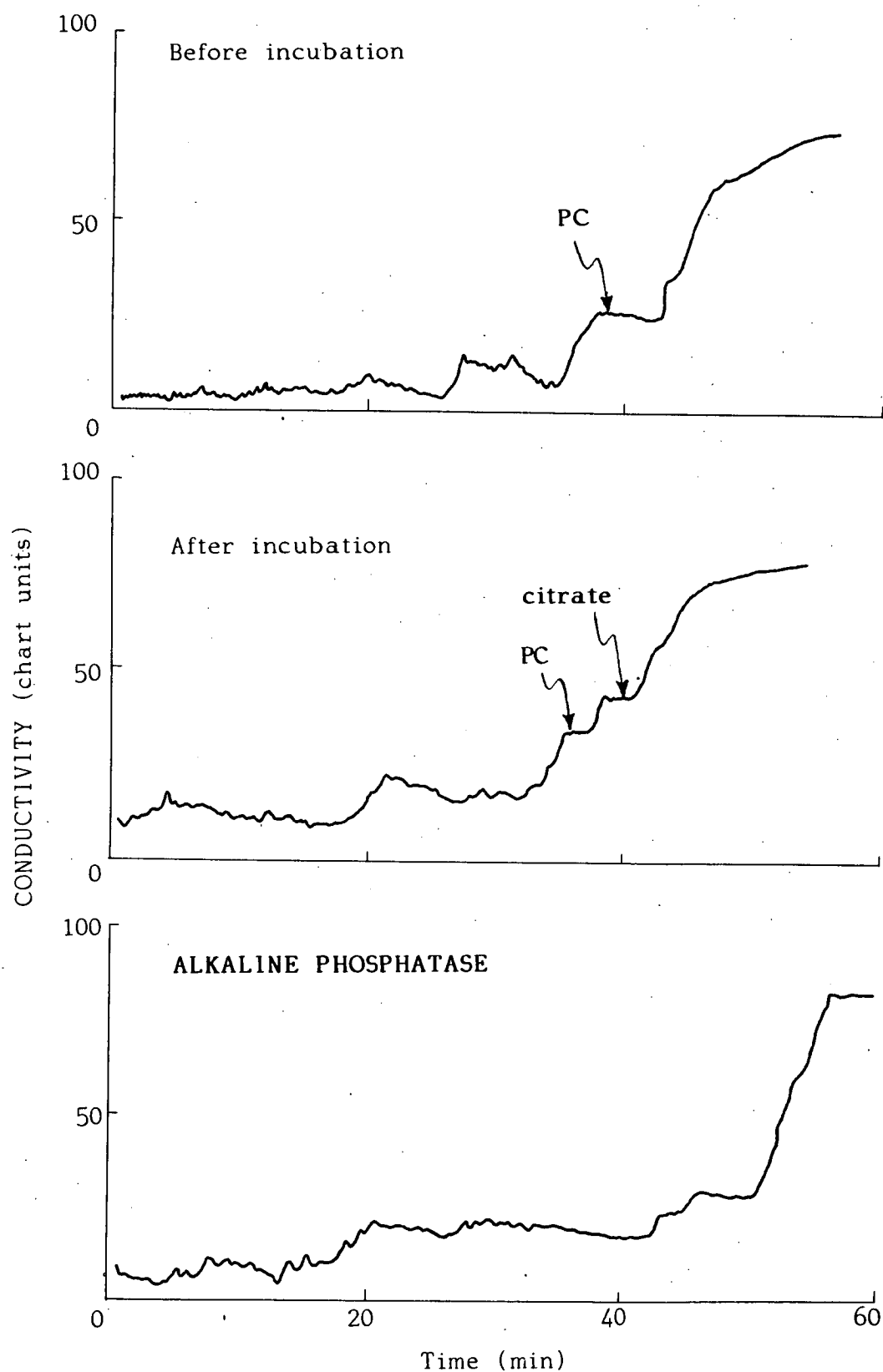
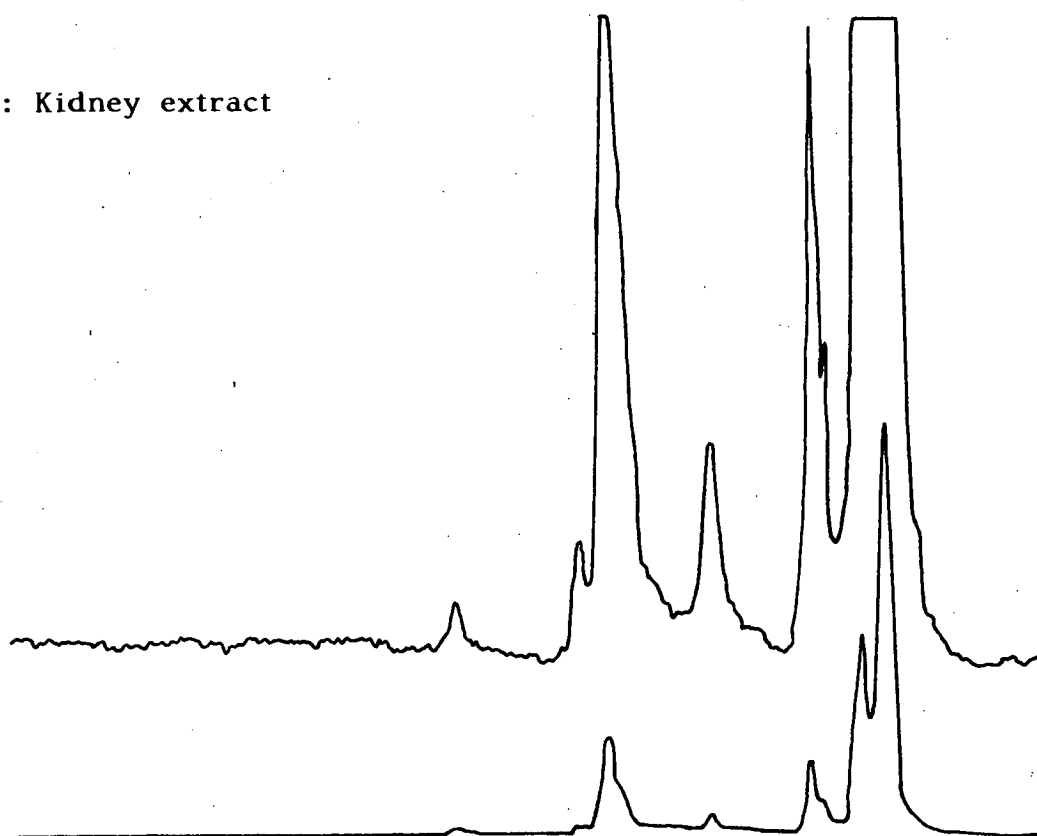


Figure 6.7 ^{31}P NMR spectra of rat kidney extracts in the presence or absence of added PC.

a: Kidney extract



**b: Kidney extract
+ PC standard**

GPE = phosphatidyl
ethanolamine

GPC = phosphatidyl
choline

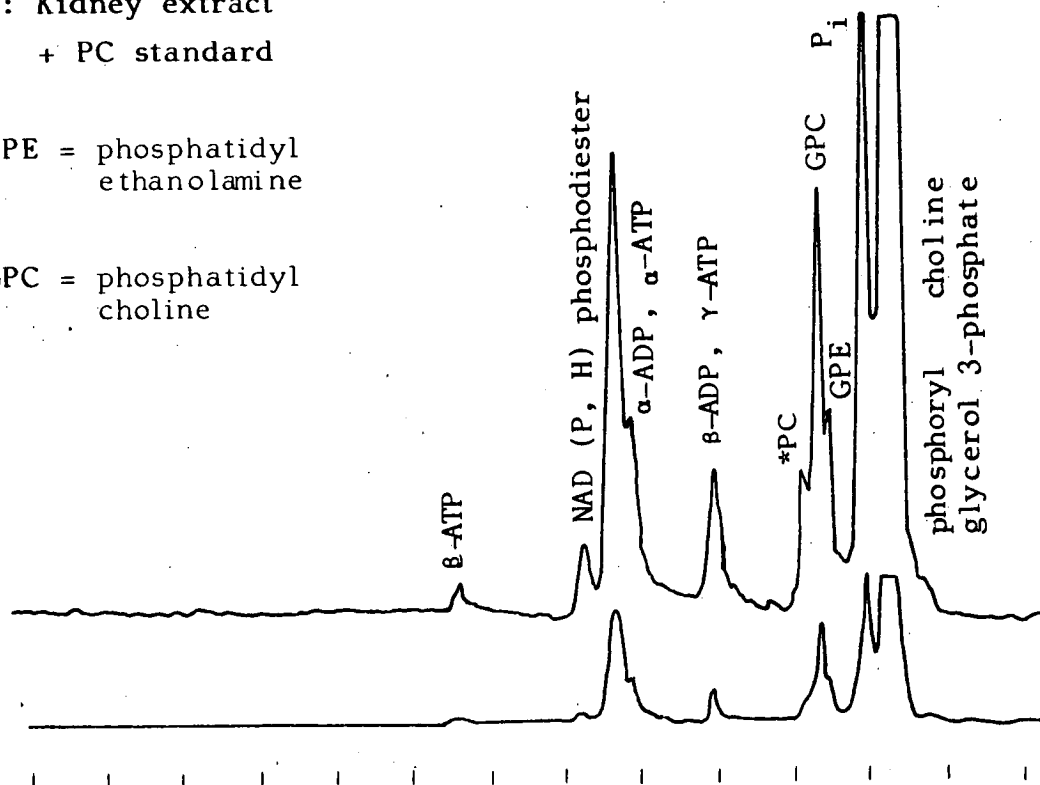
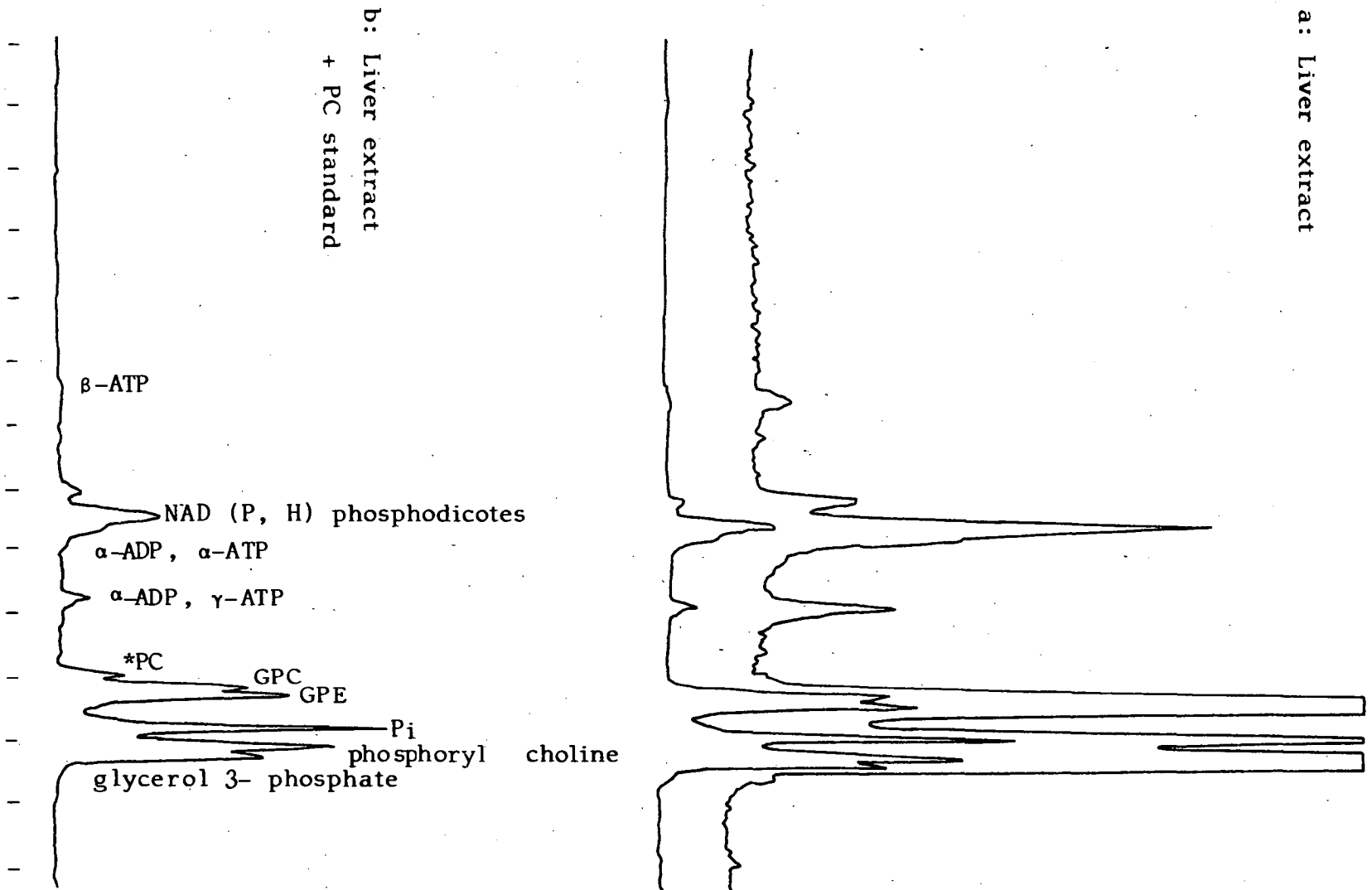


Figure 6.8 ^{31}P NMR spectra of rat liver extracts in the presence or absence of added PC.



presence will be masked by other, more abundant, phosphoric esters.

Sargeant and Stinson (1979) have demonstrated that vanadate is a potent phosphatase inhibitor. Therefore in one experiment 1mM vanadate was added in an attempt to minimise any hydrolysis of PC. The homogenate was deproteinised and analysed in an identical way to a sample without added vanadate. However, no peak due to PC was observable, again emphasising the need to concentrate PC before spectroscopic analysis.

6.4 DISCUSSION

The data presented demonstrate that PC occurs naturally. Previous work by Lehninger (1977) and Howard (1976) had failed to identify definitively the compound(s) responsible for the potent anti-calcifying ability they observed in biological fractions. In contrast, all of the analytical techniques employed here confirmed the identity of the isolated material as PC. Such a finding was possible only after preparation of authenticated PC and demonstration that separation by an ion-exchange chromatography at neutral pH does not lead to artefact formation of PC.

Further studies indicated that a quantitative recovery of PC after deproteinisation of biological samples was possible allowing investigation of biological extracts. Perchloric acid extracts from various tissues, selected due to their importance in Ca^{++} metabolism, and mitochondria from soft tissues, were subjected to ion-exchange chromatography giving a peak containing hydrolysable citrate. Significantly, the peak eluted

at a volume characteristic of PC. In order to ensure that this compound was indeed PC, biological fractions were subjected to further analysis.

The coupled enzymatic assay indicated that citrate could only be detected in the biological samples after pre-incubation with alkaline phosphatase. Although citryl phosphate (i.e. phosphate esterified to a carboxyl group) might meet enzyme requirements, stability data eliminate this possibility as the compound relative to PC is short-lived (see Section 2.5 for details). In additional tests PC from biological extracts co-electrophoresed and co-chromatographed with a reference standard of PC. A phosphate staining spot with the same R_F as authentic PC was eluted under the conditions and shown to contain citrate. Analytical isotachopheresis indicated that not only did the biological samples contain a component with the same mobility as PC, but when incubated with alkaline phosphatase the zone decreased in width and citrate was released. Thus the approach adopted using a variety of techniques to characterise PC has enabled unambiguous demonstration that PC occurs naturally in whole tissue (kidney, intestine and liver) and mitochondria (liver - 0.4, kidney - 0.8, intestine - 1.2nmol PC/mg mitochondrial protein).

Whereas the identification of PC in whole tissue and mitochondria is assured, PC has only been tentatively identified in bone and urine based on its eluting volume from ion-exchange columns and chemical citrate analysis. With bone the avid binding of PC to hydroxyapatite has hampered studies and it may even be that, like the nucleotides

(Krane and Glimcher, 1969), PC is decomposed by the HAP catalytic surface. Despite these problems a qualitative indication that PC occurs in bone was obtained.

In urine, the high concentration of salts made investigation difficult since a large amount of resin was required to bind only a relatively small amount of urine. Such an approach was adopted because the usual method used to separate salts from urinary metabolites was inapplicable. In this method barium was used to precipitate the unwanted anions, but it was found that PC co-precipitated or was adsorbed onto the precipitated material. Nevertheless, even omitting the barium precipitation step from the procedure, PC, albeit at a very low concentration, was observed in urine. As another consideration, urine does contain phosphatase activity so the possibility arises that PC may be present initially at high concentration in the kidney tubules. However, due to the time of passage of urine, and collection, storage and filtration of the samples, it is possible that only a low concentration is then present after hydrolysis in vivo and during the work-up of the samples.

Although PC has been identified in biological extracts using a range of techniques, a further test using ^{31}P NMR spectroscopy was unable to confirm its presence for two reasons: (i) the PC resonance was obscured by the resonances from phospholipids, and (ii) the PC concentration was probably too low for detection without prior purification. Thus ion-exchange chromatography is indicated but even after this it may be necessary to adjust the pH to shift the PC resonance away from phospholipid resonances (see

Section 2.4.3.1.3) which are independent of pH (Seeley, pers. comm.). However, due to the unique features of the ^{13}C NMR spectrum of PC, it may be that ^{13}C NMR spectroscopy could be the most useful of the spectroscopic techniques for characterising PC in vivo. The low natural abundance of ^{13}C indicates that it would be necessary to process at least 100 pairs of rabbit kidneys to obtain 1-5mg of material sufficient for spectroscopic purposes. If, however, rabbit chow was supplemented with a [^{13}C]-labelled compound, less rabbits would have to be used since PC might be isotopically-labelled allowing ^{13}C NMR on smaller amounts of extracted material. For mitochondrial investigations into the biosynthesis of PC, ^{13}C NMR of mitochondria incubated in the presence of a ^{13}C -labelled substrate could prove useful because of the unique ^{13}C NMR spectrum of PC. In order to transfer a suitable PC substrate (e.g. acetate) into mitochondria [^{13}C]-acetyl carnitine added to the incubation may be useful.

Even without these spectroscopic data, analysis indicates that PC does indeed occur naturally; this, together with its recognised physico-chemical properties, establishes it as a new factor for consideration in the control of biological calcification. It is consequently valuable to discuss how its presence at various sites relates to possible roles it might have physiologically.

When calcium phosphate granules are deposited in mitochondria they form as the amorphous rather than crystalline state. This amorphous phase is more labile (Termine and Eanes, 1972) and soluble (Meyer and Eanes, 1978a) than HAP so that Ca^{++} stores are more readily mobilised by

mitochondria, a factor crucial to the intracellular handling of Ca^{++} . Because of its mitochondrial occurrence and physico-chemical properties, PC could have a role in stabilising this amorphous form.

The possible presence of PC in bone suggests it may be involved in the mineralisation process. However, what must be determined is whether PC is bound to HAP before it is deposited in bone or whether its presence merely reflects a high affinity of PC for HAP, so that PC in the extracellular fluid is removed from the circulation by HAP. In the former case, it may be that PC stabilises the mitochondrial micro-packets of ACP proposed by Lehninger (1970) to be the source for the HAP of bone.

The proposed roles of PC all centre on its ability to inhibit calcification. It is possible then that its amounts in mitochondria from soft tissue (intestine > kidney >> liver) reflect the amount of Ca^{++} flux in that tissue and a need to maintain a labile store of calcium phosphate in the mitochondria of those tissues. Further, its presence in kidney indicates it may be secreted into urine and, in fact, PC has been tentatively identified there. At this site PC could play a role in inhibiting lithiasis since it inhibits both calcium oxalate and calcium phosphate formation and exerts a potentiating effect on the inhibitory ability of artificial urine.

The possible role of PC in urine to inhibit lithiasis indicates that a valuable study could be to determine the levels of PC in the urine of stone-formers and to compare this with the levels found in the urine of healthy individuals.

Such a study could be facilitated by employing one of the unique characteristics of PC, namely its adsorption onto HAP, to afford preliminary separation of PC from interfering salts (e.g. SO_4^{2-} , Cl^- , P_i^{2-}) which are only slightly adsorbed. Before this approach could be adopted, however, it would be necessary to devise a method to recover PC from HAP.

Although data in this chapter have demonstrated that PC is found at three key sites of Ca^{++} homeostasis - kidney, intestine and bone - and have allowed a preliminary estimation of its concentration in mitochondria from soft tissue, many more avenues of research have been opened up. The questions posed could be answered by studies along two lines. First, investigation of (a) the distribution of naturally-occurring (endogenous) PC, and (b) the fate of administered (exogenous) PC, at the subcellular level which could aid definition of the biological role(s) of PC, its potential for clinical use, and its likely site(s) of biosynthesis. Second, detection of PC from diverse biological sources, including biological calcifying systems might allow discussion of its physiological role(s) and interspecies comparisons. Biological calcifying systems worth investigating include tendon calcification, worm calciferous gland, hen egg shell gland, molluscan veliger development, and slime moulds (Althoff et al., 1978; Yamada, 1976). As an extension of these types of study, the effect of added PC on calcification could also be studied.

6.5 SUMMARY

1. TCA and HClO_4 extraction techniques quantitatively recovered PC.

2. PC was detected by TLC, electrophoresis, isotachophoresis, and enzymatic and chemical citrate assays in purified fractions of biological extracts.

3. PC was found in rat and rabbit kidney and liver, rat intestine. Preliminary experiments indicated PC might occur in rabbit bone and human urine.

4. Soft tissue mitochondria contain PC at a level of ca. 1nmol/mg mitochondrial protein depending on the source of the mitochondria.

5. Intestinal mitochondria > renal mitochondria > liver mitochondria as sources of PC.

6. ^{31}P NMR failed to detect PC in impurified biological extracts. Phospholipids may have obscured the PC signal.

CHAPTER 7

IN VIVO LABILITY OF PHOSPHOCITRATE

7.1 INTRODUCTION

The natural occurrence of PC raises many interesting questions, for example, (i) is it involved in controlling mitochondrial calcification, (ii) is it a metabolic substrate, (iii) does it act as a phospho-donor in intermediary metabolism, (iv) does it have a role in urolithiasis, and (v) can its physico-chemical properties be exerted in vivo as a controlling influence on calcification? The last question has important clinical ramifications since if PC is stable in vivo, advantage could be taken of its anti-calcifying properties. PC could be of potential use when it is desirable to inhibit Ca^{++} salt deposition (or dissolution) as in the treatment of, for example, Paget's disease, osteolytic sarcoma or urolithiasis.

An attempt has been made to obtain data that might answer some of these questions by taking advantage of the availability of [^{32}P]-PC (Chapter 2). This present chapter then reports on the fate of intravenously injected [^{32}P]-PC, in particular its tissue distribution and lability. Comparative experiments using an injection of [^{32}P]- P_i - the labelled hydrolysis product of [^{32}P]-PC - are also reported since enzymatic cleavage of PC is expected in vivo.

7.2 METHODS

7.2.1 ADMINISTRATION OF [^{32}P]-PC AND [^{32}P]- P_i INTO RATS: COLLECTION AND TREATMENT OF SAMPLES

The fate of administered radioisotopes was studied in male 170-180g Wistar rats. A solution of 1mg ($0.5\mu\text{Ci}$) of either sodium [^{32}P]-PC (specific activity $200\mu\text{Ci}/\text{mmol}$; prepared as described in Section 2.4.2) or [^{32}P]- P_i (specific activity $70\mu\text{Ci}/\text{mmol}$; obtained from Radiochemical Center, Buckinghamshire, Amersham, England) was administered intravenously through a tail vein to a rat lightly anaesthetized with ether. At designated times rats were exsanguinated, the blood collected in heparinized tubes and immediately frozen in liquid nitrogen. Simultaneously the body cavity was opened; kidneys, intestine and liver were excised, freeze-clamped and stored in liquid nitrogen. Bone from fore and hind limbs was taken as quickly as possible; the cartilagenous ends were removed and the marrow blown out. The demarrowed bone samples were stored in liquid nitrogen.

In one set of experiments, ureters were ligated prior to the injection of the radioisotope and, at the conclusion of the experiment, the bladder and its contents were retained and kept frozen.

7.2.2 MEASUREMENT OF TOTAL COUNTS

Total [^{32}P]-radioactivity present in tissues was assessed by the method of Mahin and Lofberg (1966). Blood ($200\mu\text{l}$), urine ($200\mu\text{l}$) or powdered soft tissue (200mg) or bone (150mg) samples were treated with $200\mu\text{l}$ of 60% HClO_4 and $400\mu\text{l}$ of 30% H_2O_2 , before sealing the tubes with

tops resistant to digestion conditions. The tubes were heated either at 30–60°C for blood and urine samples, or 70–80°C for tissue samples. The samples were digested for 1–2h until dissolved and the liquid was colourless or only faintly discoloured.

Scintillation fluid [18ml of a mixture of 0.6% 2,5-diphenyloxazole (PPO) in toluene, and cellusolve (10:6) v/v] was added to each of the samples and the vials counted.

7.2.3 TCA EXTRACTION OF SAMPLES

The recovery of radiolabelled metabolites from tissue samples for subsequent analysis involved the dropwise addition of ice-cold trichloro acetic acid (TCA) to vigorously stirred samples. One ml of 12% TCA was added to blood samples (1ml), whereas 2ml of 6% TCA was added to tissue samples (<800mg). The denatured protein was removed by centrifugation and the supernatant was retained and shaken horizontally for 10–15 min, with 4ml of a solution of tri-n-octylamine in Freon (Refrigerant R11; 8.8ml to 50ml). The tubes were centrifuged at 100g for 5 min to clarify the neutral aqueous layer which was then removed and lyophilised before analysing for inorganic phosphate and running electrophoretograms.

7.2.4 ANALYSIS OF INORGANIC PHOSPHATE

Organic phosphoric esters were separated from inorganic phosphate essentially by the method of Ohnishi (1978) using a stationary support of polyvinyl pyrrolidene (PVPP) to adsorb the phosphate-molybdate complex, allowing unbound phosphoric esters to pass through.

7.2.4.1 Preparation of PVPP columns

PVPP (10g) was washed twice with 500ml volumes of 0.5M HCl then twice with water. The suspension was filtered and the PVPP air-dried overnight. Powdered PVPP (1g) was then suspended in 10ml of water and, as it was being vigorously stirred, 0.5ml portions were taken and transferred into cotton-wool plugged, 2ml tuberculin syringes. The syringes were supported in test tubes enabling collection of the eluant. The tubes were centrifuged at 200g for 5 min before the addition of 1ml of water and another centrifugation at 200g for 5 min.

7.2.4.2 Solutions required for column operation

Solutions required included: 5% ammonium molybdate (w/v) filtered before use (AMB reagent), 250mM formate adjusted to a pH of 3.0 with HCl (formate buffer), 0.5M NH_4OH (eluting solution), 3M Tris solution, 0.5M Tris-HCl buffer, pH 7.4, and scintillant (that described above, 7.2.2).

7.2.4.3 Column operations

Washing solution (0.75ml of 1:3:1 H_2O /AMB reagent/formate buffer, v/v, prepared daily) was added to the tops of the columns and these were centrifuged at low speed (8g) for 5 min. The lyophilised TCA extracts were each dissolved in 0.3–0.5ml water. From this 0.25ml was taken and added to 0.75ml of AMB reagent and 0.25ml of formate buffer before thorough mixing. A 1ml sample of this solution was taken and transferred carefully to the top of a PVPP column in a tuberculin syringe, ensuring no air-locks formed.

Low speed (8g) centrifugation for 5 min left [^{32}P]- P_i adsorbed as a yellow band on the PVPP column. Residual organic phosphoric esters were recovered with 0.5ml of the washing solution. Combined eluant and washing were added to a counting vial and 18ml of scintillant added. Also counted was an aliquot of the untreated sample allowing calculation of the percentage of the total count due to organic phosphoric esters.

Elution of adsorbed [^{32}P]- P_i from the column was effected by transferring the columns into new test tubes and adding 0.75ml of the eluting solution before centrifugation at 200g for 10 min. The eluant was transferred into a counting vial and 0.85ml of 0.5M Tris-HCl buffer (pH 7.0) was added, followed by scintillant. The process was repeated and the sum of the radioactivity in the two vials gave the counts due to [^{32}P]- P_i .

Appropriate blanks were included enabling the background to be counted. In this way the proportion of the total counts due to inorganic phosphate or phosphoric esters could be determined.

7.2.5 DETERMINATION OF [^{32}P]-PC

The proportion of the total counts due to [^{32}P]-PC was determined by high-voltage electrophoresis. Accordingly, lyophilised TCA extracts were dissolved in a minimum amount of water (ca. 100 μl). An aliquot (10-20 μl) was taken and counted. One half of the remainder (40 μl) was applied to Whatman 3MM paper and to the other half [^{32}P]- P_i and [^{32}P]-PC standards were added; the standards contained

the same amount of radioactivity as the biological extracts. A mixture of the extract and internal standards was then also applied. This procedure ensures that the reference standards are present in a quantity that does not obscure the counts in the biological standard nor is insufficient for observation.

Separation of radiolabelled components was effected by electrophoresis (Section 3.3.2.3). After 2h the paper was cut into two sections (origin to 17cm mark, 17cm to 37cm mark) and each section then placed in a Birkoff spark chamber for visualisation of the radioactive areas.

The level of radioactivity in each spot was subsequently quantitated in the following manner: the paper was marked in a grid fashion and cut into sections (approximately 2 x 3cm), each section was coiled to allow it to be placed in a vial supported by a thoroughly washed (0.5N HCl) fine mesh (nylon material) as shown in Figure 7.1. The paper was completely moistened with 1ml of 0.5N HCl and the lid was screwed on so that the supported coil of paper was fixed in place (see Figure 7.1). The vial was centrifuged at 100g for 5 min before another 1.0ml of 0.5N HCl was added and the process repeated.

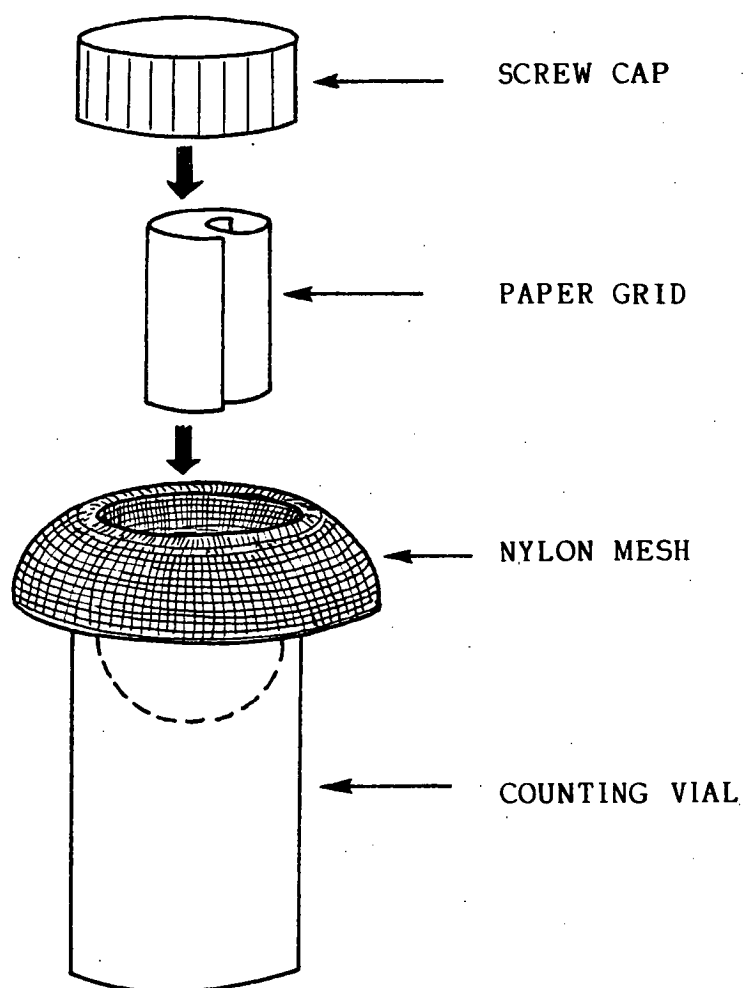
Scintillant (18.0ml) was added to the 2ml of acid extract and the radioactivity determined. Appropriate blanks were also included.

7.3 RESULTS

7.3.1 TOTAL COUNTS IN SAMPLE

Initial experiments involved excision of liver, kidney

Figure 7.1 Apparatus used to elute radio-active components from electrophoretograms.



and bone and the collection of blood at 5, 15 and 30 min after an injection of $[^{32}\text{P}]\text{-PC}$ or $[^{32}\text{P}]\text{-P}_i$. Liver and kidney weights were recorded and since the total skeletal weight and blood volume may be calculated knowing the sex and body weight of the rats used (Donaldson, 1920; Altman and Dittmar, 1974) the counts in the samples could be expressed as a proportion of the total count found in each organ.

Figures 7.2 and 7.3 give the results obtained after an injection of $[^{32}\text{P}]\text{-P}_i$ (Figure 7.2) or $[^{32}\text{P}]\text{-PC}$ (Figure 7.3). After 30 min, uptake by the tissues appeared to be almost complete in both experiments. When $[^{32}\text{P}]\text{-P}_i$ was injected, 20% of the total injected count resided in bone after 30 min, 10% in liver and <5% in kidney. However, after an injection of $[^{32}\text{P}]\text{-PC}$ more than 40% of the total count was taken up into bone followed by 15% in kidney and only 5% in liver.

To compare a wider range of biological samples, rats were killed after 30 min and samples collected as before, but in addition urine and intestine were also retained. Results are given in Figure 7.4 and indicate that intestine and urine only contain a small amount of the initial radioactivity administered (<5%).

7.3.2 PROPORTION OF $[^{32}\text{P}]\text{-P}_i$ IN BIOLOGICAL SAMPLES

From the data presented above, it is tempting to identify bone and kidney as the major sites of PC uptake. However, what is unknown is the proportions of counts due to unreacted $[^{32}\text{P}]\text{-PC}$ and the proportion due to one of its hydrolysis products $[^{32}\text{P}]\text{-P}_i$. $[^{32}\text{P}]\text{-PC}$ is totally recovered

Figure 7.2 Rate of disappearance of radio-activity in blood and its appearance in tissue following administration of $[^{32}\text{P}]\text{-P}_i$.

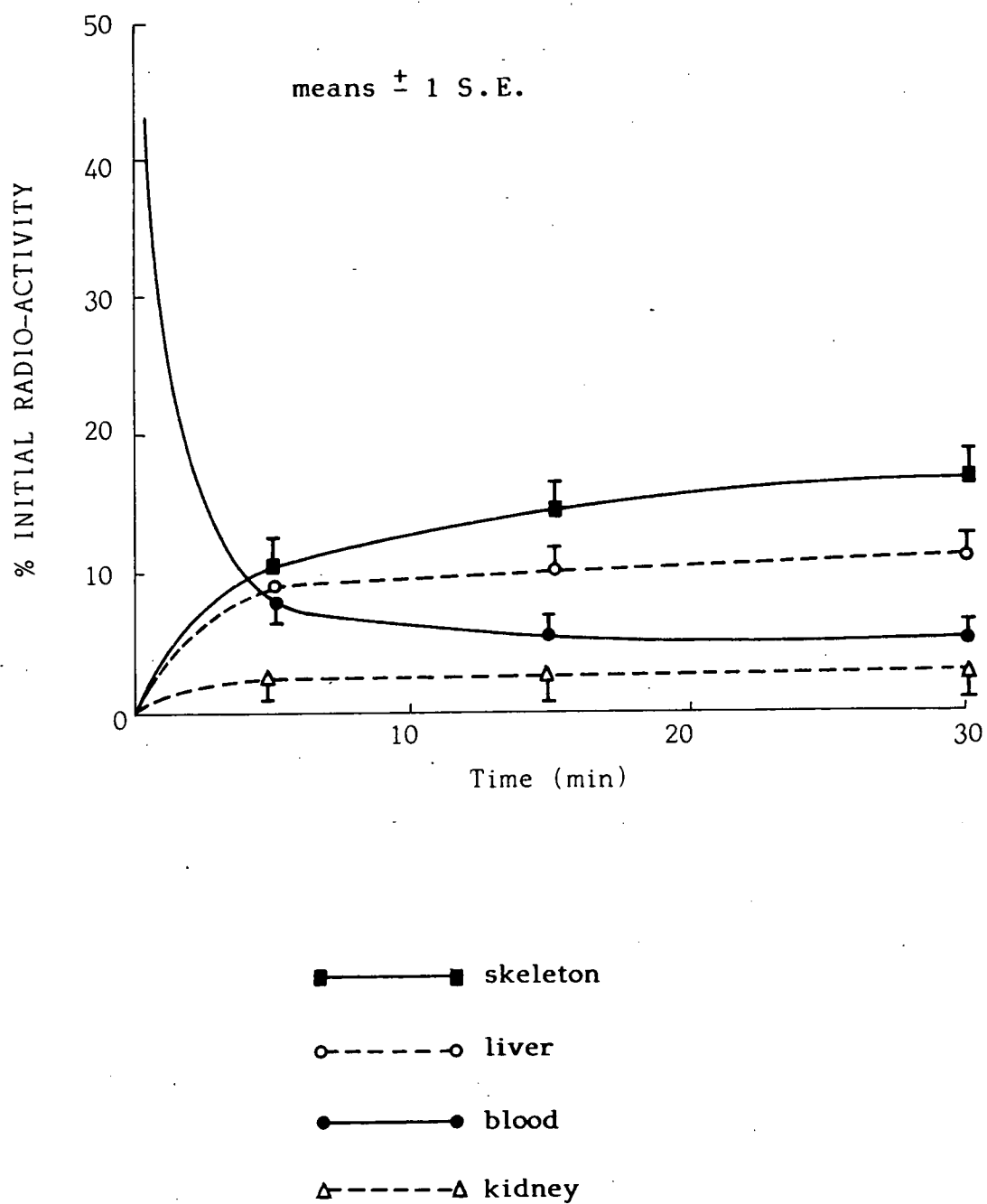
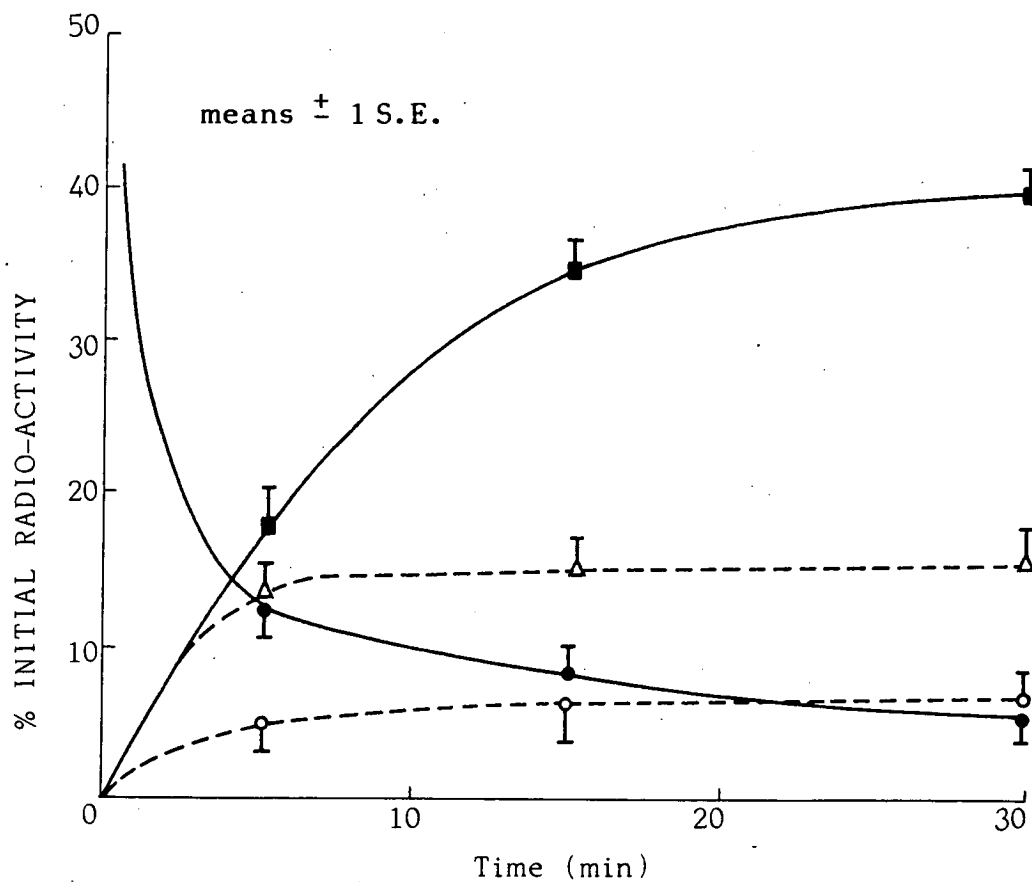


Figure 7.3 Rate of disappearance of radio-activity in blood and its appearance in tissue following administration of [^{32}P]-PC.



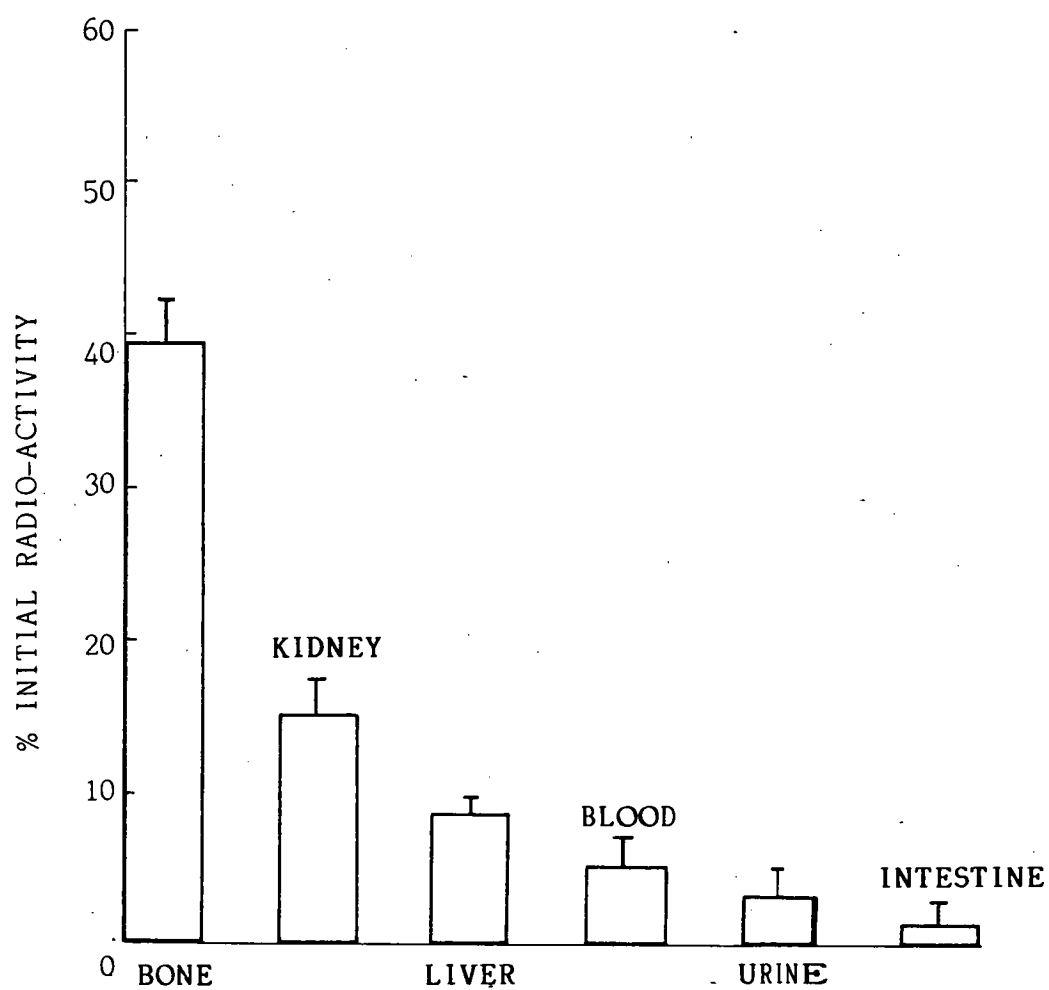
■ — ■ skeleton

○ - - - ○ liver

● — ● blood

△ - - - △ kidney

Figure 7.4 Distribution of radio-activity 30 min after administration of [^{32}P]-PC to rats.



Means \pm 1 S.E.

under conditions used to adsorb [^{32}P]- P_i onto PVPP columns so that this treatment is applicable to biological extracts. Table 7.1 gives the proportion of total counts due to organic phosphoric esters in liver, kidney and blood at designated times after an injection of [^{32}P]-PC. The data indicates that with the exception of blood, [^{32}P]- P_i was prominent in the tissues.

7.3.3 PROPORTION OF [^{32}P]-PC IN BIOLOGICAL SAMPLES

High voltage electrophoresis was used to resolve the [^{32}P]-labelled components as an initial step in determining the proportion of initial [^{32}P]-PC present. Results from these analyses are given in Figures 7.5 and 7.6 for experiments with [^{32}P]-PC and [^{32}P]- P_i respectively. [^{32}P]-PC and [^{32}P]- P_i in the samples are easily identifiable because internal standards were included.

Clearly [^{32}P]-PC remains essentially unchanged in blood even after 30 min but in contrast, only 15% of the radioactivity present in the kidney was due to PC after only 5 min; and after 30 min only a trace (<5%) of [^{32}P]-PC remained in kidney. Electrophoretic patterns obtained from liver extracts indicated that [^{32}P]-PC had been hydrolysed and a large number of phosphoric esters produced.

The comparative experiments with [^{32}P]- P_i indicated that, at least in kidney and blood, no phosphoric ester was produced which migrates with the same mobility as PC, ensuring that spots identified as [^{32}P]-PC were probably not contaminated with some compound co-electrophoresing with it.

Table 7.1 Proportion of radiolabelled compounds present as organic compounds after administration of [^{32}P]-PC.

TIME AFTER INJECTION (min)	% RADIOACTIVITY DUE TO ORGANIC PHOSPHATES*		
	BLOOD	KIDNEY	LIVER
5	95 \pm 4	40 \pm 3	53 \pm 4
15	87 \pm 3	47 \pm 6	58 \pm 6
30	78 \pm 2	38 \pm 3	45 \pm 5

*At least 3 trials; means \pm 1 S.E.

Figure 7.5 Radio-active components resolved by electrophoresis after administration of [32 P]-PC.

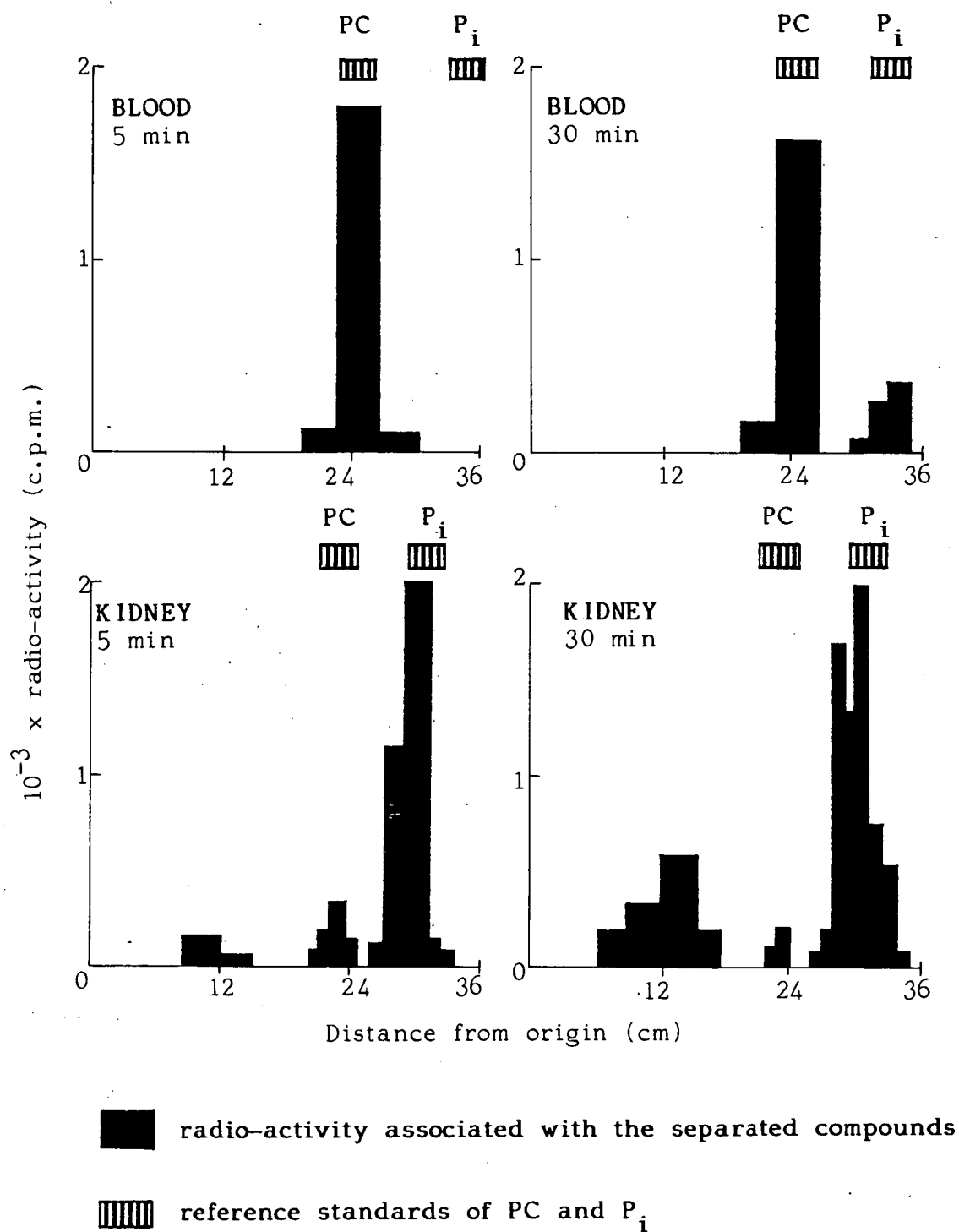
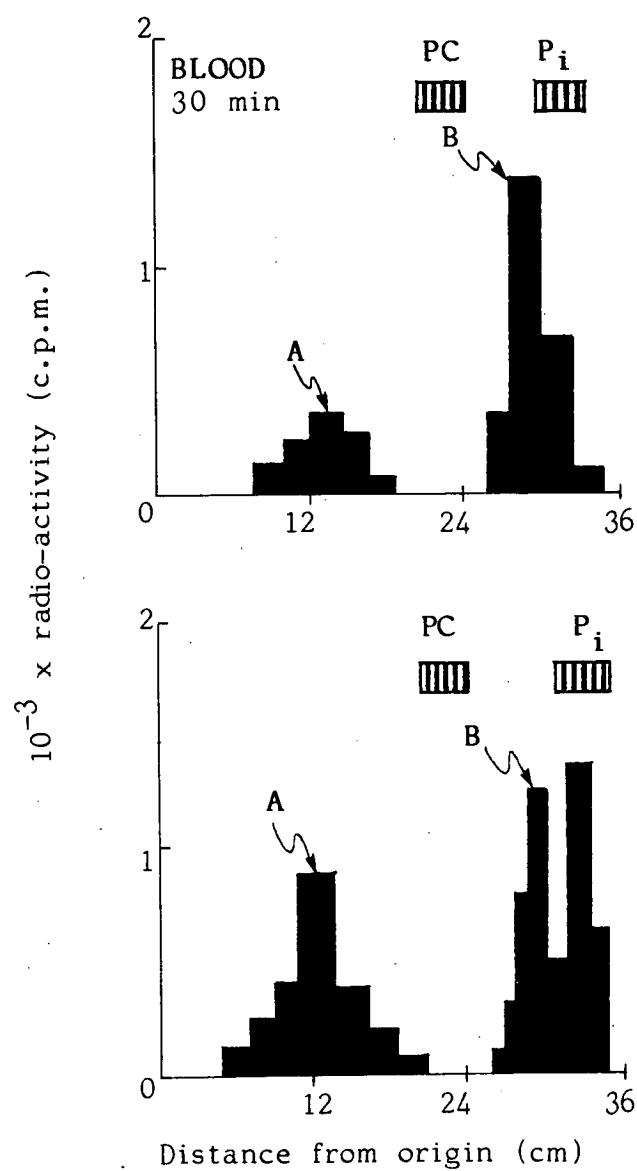


Figure 7.6 Radio-active components resolved by electrophoresis after administration of $[^{32}\text{P}]\text{-P}_i$.



■ radio-activity associated with separated compounds

▨ reference standards of PC and P_i

Detailed analysis of the electrophoretograms made it possible to distinguish whether PC was being metabolised to organic esters or whether PC was being hydrolysed to P_i which was then incorporated into organic phosphoric esters. With blood and kidney two major organic components (A and B in Figure 7.6) were prominent after an injection of $[^{32}P]-P_i$, both apparent in kidney even after 5 min. Peaks A and B were also observed in the electrophoretic patterns from kidney extracts after an injection of $[^{32}P]-PC$ (see Figure 7.5) supporting the contention that PC was being hydrolysed to citrate and P_i before P_i became incorporated into the phosphoric esters responsible for the characteristic electrophoretic patterns observed.

In urine (Figure 7.7) no PC was detectable after 30 min. Figure 7.8 shows the electrophoretic pattern obtained after a 30 min incubation of $[^{32}P]-PC$ with urine in vitro at $37^\circ C$. In the latter instance very little hydrolysis occurred. These observations are consistent with the rapid hydrolysis of PC occurring at the kidney site before passage into urine.

7.4 DISCUSSION

An understanding of the metabolism of administered PC could provide an insight into the physiological roles of this compound. It is clear from the data presented that radioactivity from an intravenous injection of $[^{32}P]-PC$ was widely distributed throughout an animal after 30 min. Time course studies indicated uptake by the tissues was essentially complete after 30 min, blood being almost completely depleted of $[^{32}P]-PC$, whereas the radioactivity was concentrated

Figure 7.7 Radio-active components resolved by high-voltage electrophoresis after administration of [^{32}P]-PC.

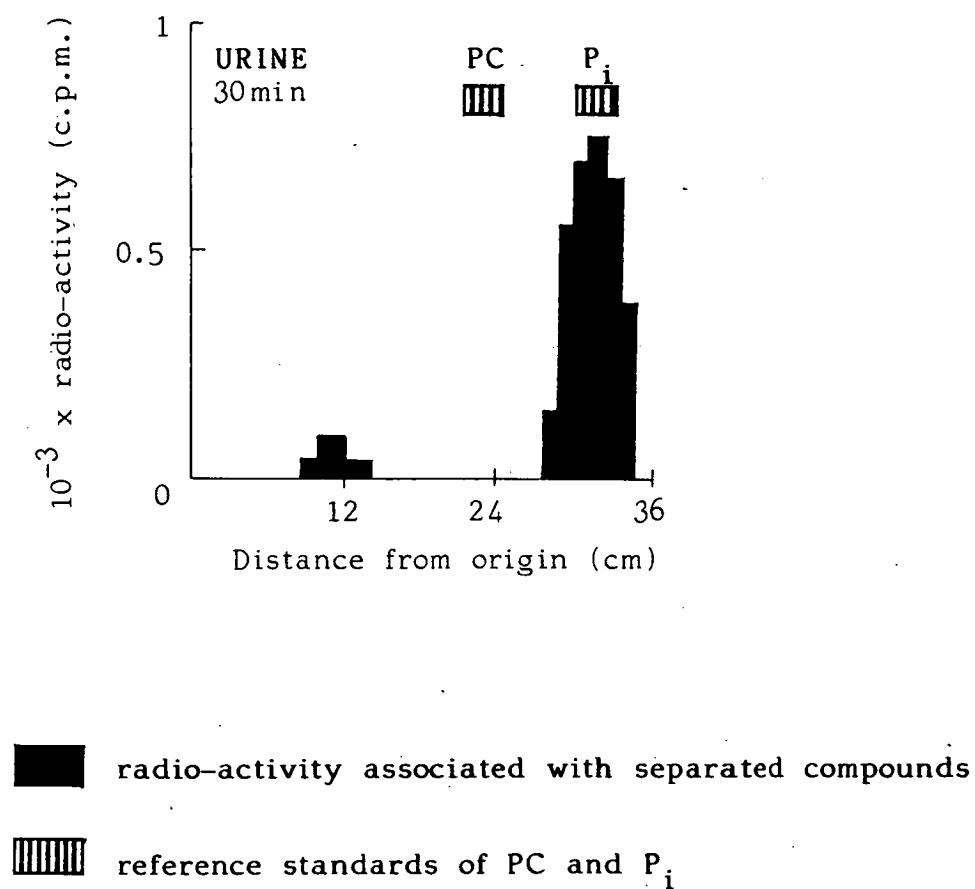
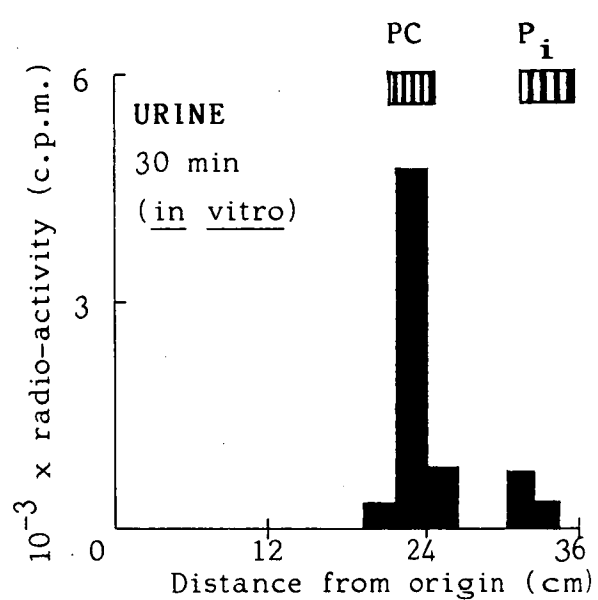




Figure 7.8 Radio-active components resolved by high-voltage electrophoresis: [^{32}P]-PC incubated with urine in vitro.



 radio-activity associated with separated compounds
 reference standards of PC, P_I

mostly in bone (ca. 40%) or the kidney (ca. 15%).

The rapid uptake of [^{32}P]-PC into bone is consistent with the demonstration in vitro that PC was avidly bound by HAP (Chapter 5). However, direct evidence that it is PC bound to bone is lacking due to difficulty in eluting PC from HAP. Nevertheless, the uptake of counts into bone is likely to be a true reflection of PC uptake since (1) only ca. 20% of the hydrolysis product of [^{32}P]-PC, namely [^{32}P]- P_i , is adsorbed onto bone, and (2) [^{32}P]-PC remains essentially unchanged in blood.

Another important site for the uptake of [^{32}P]-PC is the kidney where PC is rapidly hydrolysed. This contrasts to its behaviour in blood where it remains essentially unchanged even though blood is rich in phosphatase activity. In the kidney, the lability of PC suggests that special conditions may prevail there for phosphatase action, or that phosphatases there have a special propensity to hydrolyse PC, or even that a specific PC cleavage enzyme exists which rapidly inactivates the molecule. The degradative process for PC at the kidney site therefore warrants further study.

The lability of administered (exogeneous) PC in kidney is relevant to understanding the role of endogeneous (naturally occurring) PC. Despite the lytic activity demonstrated by the kidney, PC is detectable in kidney mitochondria. PC should be completely hydrolysed in the time it takes to prepare mitochondria so these observations suggest that mitochondrial compartmentalisation must act to shield endogeneous PC from the lytic activity in the extramitochondrial compartment. Perhaps inactivation of the molecule is necessary

should it leak out of the mitochondria where its potent anti-calcifying properties ensure HAP needles do not form. It is important then to gain further information on the intracellular distribution of PC and to elucidate the biological site(s) of synthesis and degradation.

The lability of PC in kidney explains why, when [^{32}P]-PC is injected, only [^{32}P]- P_i is observed in urine after 30 min, in contrast to the stability that [^{32}P]-PC exhibits when incubated in urine in vitro. In fact analysis of the electrophoretograms indicated that the hydrolysis of [^{32}P]-PC into [^{32}P]- P_i and citrate was the major in vivo reaction. This fact militates against a role for PC as a phospho-donor in intermediary metabolism.

The data also allow consideration of the possible therapeutic usefulness of PC apart from discussion on the physiological properties of PC. PC is stable in blood which may be a property making PC useful in the prevention of calcium deposition associated with arterial calcification. Analogously, the diphosphonates, less potent inhibitors of calcification than PC, are known to inhibit arterial calcification (Fleisch et al., 1965), indicating the likely applicability of PC.

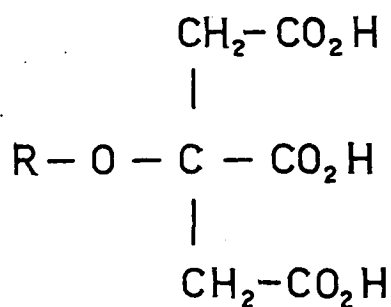
In some instances, it is necessary to have a compound that will bind to bone decreasing bone turnover, for example in the treatment of Paget's disease or osteolytic sarcoma. Studies have indicated that although diphosphonates, inert analogues of pyrophosphate, will inhibit urolithiasis (Ohata and Pak, 1973, 1974) and decrease bone turnover (Russell et al., 1980; Khairi et al., 1977) prolonged treatment leads

to many undesirable side-effects (Russell et al., 1981). Certainly they are not recommended for treating urolithiasis (Bone et al., 1979). Therefore, moderate lability of a therapeutic agent appears desirable, and PC may be useful in decreasing bone turnover without long-term side-effects.

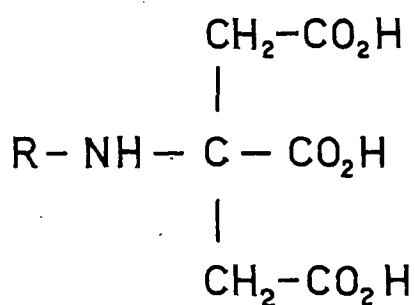
The lability of PC in the kidney makes it doubtful whether it could be of therapeutic usefulness in the prevention of lithiasis. It may be, however, that an analogue of PC stable to enzymatic hydrolysis but possessing its physico-chemical properties is more desirable. The analogues of PC that may be of potential and are predicted to be inhibitors of HAP formation based on their structures, are given in Figure 7.9. Based on knowledge of the behaviour of pyrophosphate, ATP and ADP and their analogues (Yount et al., 1971; Yount, 1974; Wilkes et al., 1973; Meyers et al., 1963), the expected order of lability of PC and its analogues is: $P-O-C > P-N-C \gg P-C-C = 0$. Consequently the "P-C-C" analogue may not be therapeutically useful due to its inertness. In the treatment of urolithiasis and perhaps skeletal disorders, the "P-N-C" analogue is indicated as a potentially useful therapeutic agent.

The synthesis of the "P-N-C" analogues could be achieved using either of the two phosphorylating techniques described in this thesis - o-phenylene chlorophosphate or 2-cyanoethyl phosphate - once "amino-citrate" is prepared. In fact the extra reactivity that amino groups have in these coupling reactions (Bodanszky and du Vigneud, 1959) should increase the yield available using the methods. However,

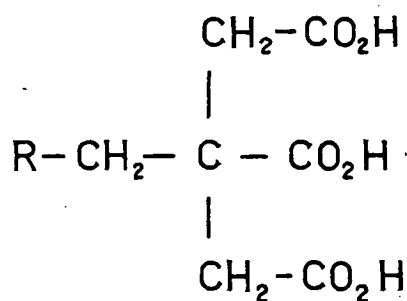
Figure 7.9 Structures of phosphocitrate and its analogues



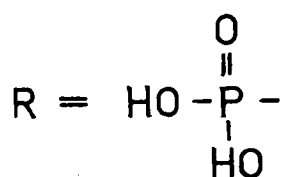
Phosphocitrate



N-analogue of phosphocitrate



C-analogue of phosphocitrate



no such coupling reactions are possible with "methyl citrate" due to the inertness of methyl groups in such reactions.

The information presented in this chapter is not only of use in elucidating the possible biological roles of PC and indicating potential clinical utility, but also for indicating future research directions. These include: (1) the use of [^{32}P]-PC or $^{99\text{m}}\text{Tc}$ -PC as a bone-imaging agent (Rohlin, 1976; Rohlin and Hammarstrom, 1976), (2) elucidation of the factor(s) responsible for the difference in the lability of PC in blood and kidney, (3) evaluation of the use of PC in the prevention of arterial calcification and to decrease bone turnover, and (4) synthesis of PC analogues and assessment of their (a) anti-calcifying ability, (b) lability in vivo, and (c) uses in therapy.

7.5 SUMMARY

1. Intravenously administered PC is taken up specifically by bone (40%) and kidney (15%).
2. PC is rapidly hydrolysed in kidney but is stable in blood and, in vitro, urine.
3. PC is degraded in vivo to P_i and citrate and does not appear to act as a phospho-donor, or to be metabolised directly to other phosphoric esters.
4. Endogeneous PC is not hydrolysed because its location in mitochondria protects it from the lytic activity in the cytosol.
5. In therapy, if advantage of the physico-chemical properties of PC is to be taken, then analogues stable to enzymatic degradation must be synthesised.

CHAPTER 8

THE SUBCELLULAR DISTRIBUTION AND BIOSYNTHESIS OF PHOSPHOCITRATE

8.1 INTRODUCTION

Fundamental roles for PC in vivo can certainly be envisaged based on its physico-chemical properties and its demonstrated natural occurrence in association with mitochondria. One role involves stabilising the calcium phosphate granules that form in mitochondria as the amorphous rather than crystalline form. The proposed action of PC consequently affects the intracellular handling of Ca^{++} and, conceivably, could be modified and extended in specific tissues. Alteration could be associated with the degree of Ca^{++} flux in a tissue (for example, intestine) or a further need to closely regulate Ca^{++} deposition (i.e. bone).

Mitochondria appear to be a likely site for the biosynthesis of PC since it has been found in this subcellular organelle. If, however, PC were present in other cellular compartments then other roles, and indeed other sites for its biosynthesis, require consideration. Clearly information is needed on the subcellular distribution of PC so that some of the questions posed can be investigated more thoroughly. This chapter describes approaches taken to gain such data and also reports experiments concerning the biosynthesis of PC.

8.2 METHODS

8.2.1 STUDIES ON THE LABILITY OF PC IN TISSUE HOMOGENATES AND METHODS OF INHIBIT PHOSPHATASE ACTIVITY

Tissue homogenates (10ml of 250mM sucrose at pH 7.4/g tissue wet weight) were prepared in a Potter-Elvehjem homogeniser using three strokes of a Teflon pestle. For lability studies [^{32}P]-PC was added into the homogenate and at designated times aliquots were removed and deproteinised with HClO_4 (Section 6.2.3.1) and the protein-free solution electrophoresed to determine unreacted [^{32}P]-PC as described previously (Section 7.3.3).

In one group of experiments the possibility of inhibiting the in vivo hydrolysis of PC was investigated by adding vanadate to a solution of PC in the presence or absence of tissue homogenate. Deproteinisation and electrophoresis of the protein-free samples was then undertaken.

In another approach, phosphatase activity in tissue homogenates was determined in the presence or absence of an inhibitor selected from a range of known inhibitors of the enzyme. Phosphatase activity was determined at pH 7.4 using p-nitrophenyl phosphate (p-NPP) as the substrate (Section 2.4.3.2.2).

8.2.2 SUCCINATE DEHYDROGENASE ACTIVITY

Succinate dehydrogenase activity is restricted to mitochondria so that this enzyme can be used to determine the mitochondrial content of biological preparations. Accordingly, either 50 μl of homogenate or 100 μl of a mitochondrial suspension was added to 400 μl of sodium phosphate buffer

(0.2M, pH 7.4), 300 μ l of sodium succinate (0.2M, pH 7.4) and 100 μ l of 1% MTT [3, (4,5-dimethyl thiazole), 2,5-diphenyl tetrazolium bromide]. The mixture was incubated at 37°C for 10-20 min before the reaction was terminated by the addition of 0.5ml of 1% trichloroacetic acid and then 4ml of tetrahydrofuran was added.

Tube contents were vigorously mixed before reading absorbance at 550nm. Tubes were read against control tubes prepared identically with the exception that water was substituted for succinate.

8.2.3 DETERMINATION OF THE SUBCELLULAR DISTRIBUTION OF PC

The subcellular distribution of PC was initially investigated by analysing mitochondria and whole tissue for PC so that the degree of PC localisation in mitochondria could be determined. Information so gained indicated whether or not mitochondria were the major source of PC.

Three rabbits were killed and one kidney from each excised, freeze-clamped and weighed. The remaining kidneys were rapidly excised, weighed and homogenised in buffered 250mM sucrose (pH 7.4). The volume of the homogenate was recorded and a sample taken and analysed for succinate dehydrogenase activity. The remaining homogenate was subjected to a differential centrifugation to prepare mitochondria.

8.2.3.1 Whole tissue extract

Frozen kidney samples were deproteinised with 200ml of ice-cold 0.5N HClO₄ and homogenised using a top drive

homogeniser (Silversen Top Drive, Waterside, Chesham, Buckinghamshire) for 1 min. Denatured protein was removed by centrifugation at 800g for 10 min, the supernatant neutralised with concentrated KOH, and then left to stand at 0°C for 10-15 min before the KClO_4 was removed by centrifugation. In order to determine the degree of binding, 10 μl of [^{14}C]-citrate was added to the extract and 1ml aliquots were taken before and after passage through a 15 x 3cm Dowex 1-X8 (HCO_3^-) column. Measurement of the radioactivity present in the two aliquots enabled calculation of the percentage binding. Typically >99% binding was obtained.

8.2.3.2 Mitochondrial extract

The mitochondria, once isolated, were suspended in 250mM sucrose, the volume recorded, and an aliquot of 1ml taken for subsequent analysis of protein and succinate dehydrogenase activity. An equal volume of ice-cold 1N HClO_4 was added to the mitochondrial suspension and a neutral extract prepared in the usual way (Section 6.2.3.4). The extract was loaded onto a 10 x 1cm column of Dowex 1-X8 (HCO_3^-) after the addition of 5 μl of [^{14}C]-citrate to enable calculation of the percentage binding. Under these conditions >99% was achieved.

8.2.3.3 Analysis of PC in biological extracts

The larger (3 x 15cm) column was eluted with 700-800ml of 0.27M NaHCO_3 (pH 7.0-7.4): the smaller (1 x 10cm) column was eluted with 400-500ml of the buffer. The columns were then eluted with 800ml and 600ml of 0.45M NaHCO_3 buffer respectively; these fractions were collected in chilled

containers. The two fractions were decarbonated with AG 50W-X8 (H^+), and sufficient resin was added to lower the pH to 5-6. The mixture was filtered, the filtrate neutralised with dilute NaOH and lyophilised. The freeze-dried residues were dissolved in 50-100ml of water and then applied to two 1 x 10cm Dowex 1-X8 (HCO_3^-) columns. A third column was loaded with 50 μ g of synthetic PC and column outlets were connected to a single channel of a peristaltic pump (Durrum 12 channel, Model 11000, Palo Alto, California). This procedure enabled the characteristic eluting volume for PC to be determined each run.

Initially the three columns were eluted with 400ml of 0.27M $NaHCO_3$ each, and then subjected to gradient elution as described before (Section 6.2.4.2.2). The 15ml fractions that were obtained were then decarbonated and acidified with 1.8ml of 5N H_2SO_4 before hydrolysis at $100^\circ C$ for 5hr followed by lyophilisation. The dried material was dissolved in 2.5ml of warm water and transferred into a test tube. An additional amount of 2.5ml water was used to rinse out the freeze-drying flasks. The combined contents were then chemically analysed for citrate (Section 3.2.2.1.1).

8.2.4 BIOSYNTHETIC EXPERIMENTS

Three approaches were adopted in attempts to elucidate aspects of the biosynthesis of PC.

8.2.4.1 In vivo

The effect of ingestion of Ca^{++} on the PC content of intestinal mitochondria was studied since the evidence presented in this thesis so far supports a role for PC in

the Ca^{++} handling of cells, in particular mitochondria. The reasoning was that perhaps Ca^{++} -loading of this organelle triggered PC production enabling storage of Ca^{++} deposits as ACP rather than as HAP. Accordingly, four male rats (150-250g) were fasted overnight before giving 200mg calcium gluconate in 1ml of 10% sucrose orally. The rats were killed 30 min later, blood collected and intestines excised. Serum was prepared and its Ca^{++} content determined by Atomic Absorption Spectroscopy (Section 4.3.2.2). Intestinal mitochondria were prepared (Section 6.2.1.4.2) and 0.2ml samples taken for protein and Ca^{++} determination. The remaining mitochondrial suspension was deproteinised with HClO_4 , a neutral extract prepared and column chromatography undertaken as described previously (Section 6.2.4.2) for the analysis of PC content.

In control experiments rats were treated in an identical manner except that calcium gluconate was omitted from the 10% sucrose solution.

8.2.4.2 In vitro - Mitochondrial incubations

Using the same rationale as with the whole animal experiments, namely that Ca^{++} -loading might initiate PC production in mitochondria, rabbit kidney mitochondria were prepared and incubated in a medium known to cause Ca^{++} -loading and the deposition of calcium phosphate granules (Greenawalt et al., 1964). Rabbit kidney mitochondria were prepared in the usual way (Section 6.2.1.4.3), before 200-400mg mitochondria protein was suspended in Medium I (Table 8.1) (1ml/mg protein) and incubated at 37°C . At designated

Table 8.1 The composition of Medium I for calcium-loading incubated mitochondria.

SUBSTANCE	CONCENTRATION (mM)
ATP	3.0
sodium succinate	10.0
P _i	4.0
MgCl ₂	10.0
Tris-HCl (pH 7.0)	10.0
CaCl ₂	4.0

Taken from Greenawalt et al. (1964).

times (0, 15, 30 min) one-third of the incubating suspension was removed, the mitochondria sedimented, deproteinised and subjected to column chromatography before analysis of PC as previously described (Section 6.2.4).

In other experiments, Ca^{++} was omitted from Medium I and 4mM citrate substituted. This experiment was designed to investigate whether the presence of the likely PC precursors - P_i and citrate - together with an energy source, ATP, could promote mitochondrial PC production. Apart from the substitution of citrate for Ca^{++} , the experiment was conducted in an identical manner to the in vivo Ca^{++} -loading experiments.

8.2.4.3 Incubation with alkaline phosphatase

If high concentrations of a phospho-accepting compound (for example, glycerol, serine), P_i and a phospho-donor (for example, phosphocreatine, ATP) are incubated with alkaline phosphatase, then the enzyme catalyses the reverse reaction: the phosphorylation of the phospho-acceptor (Kay, 1928; Morton, 1958). The possibility that "reverse" phosphatase activity could produce phosphocitrate therefore was studied. Typically trisodium citrate (2.94g) and 126mg of NaH_2PO_4 were dissolved in 5ml water and the pH adjusted to 9.8-9.9. The tube contents were incubated at 37°C for 20 min, in the presence or absence of either 100U of alkaline phosphatase and/or 30mg phosphocreatine. After this period 5ml of 1N HClO_4 was added, the solution diluted to 50ml with distilled water and the pH adjusted to 7-8 with KOH. Following centrifugation at 1,000g for 15

min to remove the precipitate, the solutions were loaded onto 1 x 10cm columns of Dowex 1-X8 (HCO_3) and subsequently eluted and analysed (Section 6.2.4.2).

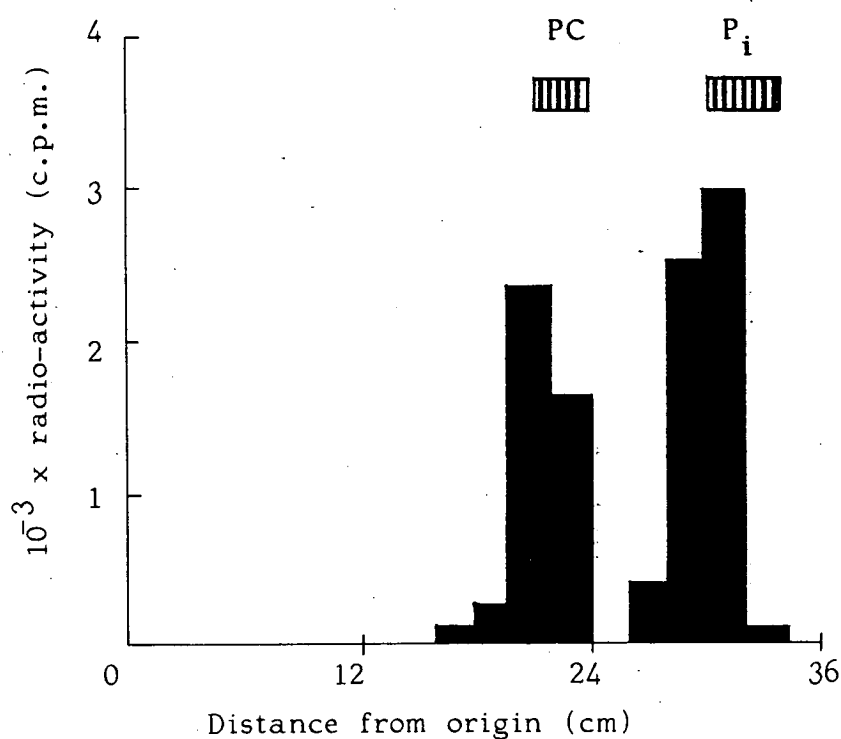
8.3 RESULTS

8.3.1 STUDIES ON THE LABILITY OF PC AND METHODS TO INHIBIT PHOSPHATASE ACTIVITY

[^{32}P]-PC added to rabbit kidney homogenates was rapidly hydrolysed within 5 min based on the disappearance of [^{32}P]-PC and the appearance of other [^{32}P]-labelled compounds as shown in Figure 8.1. This result indicated that if PC was to be detected in purified subcellular fractions a method had to be developed to inhibit the hydrolytic reactions, since procedures to separate cellular organelles from cytosol take longer than 5 min. Consequently the use of inhibitors to minimise phosphatase activity was studied.

In view of the known inhibition of alkaline phosphatase by vanadate the ability of this inhibitor to decrease the phosphohydrolytic activity in kidney homogenates was investigated. However, the addition of 5mM of vanadate into an homogenate at 0° C did not apparently significantly decrease the hydrolysis of [^{32}P]-PC (Figure 8.2). When a solution of PC was treated with vanadate (i.e. no homogenate was present), electrophoresis indicated that [^{32}P]-PC had been largely destroyed (Figure 8.3). In fact, vanadate is known to oxidise organic compounds not only under acidic conditions but also at neutral pH; this may have been the reason for the depletion of [^{32}P]-PC observed here.

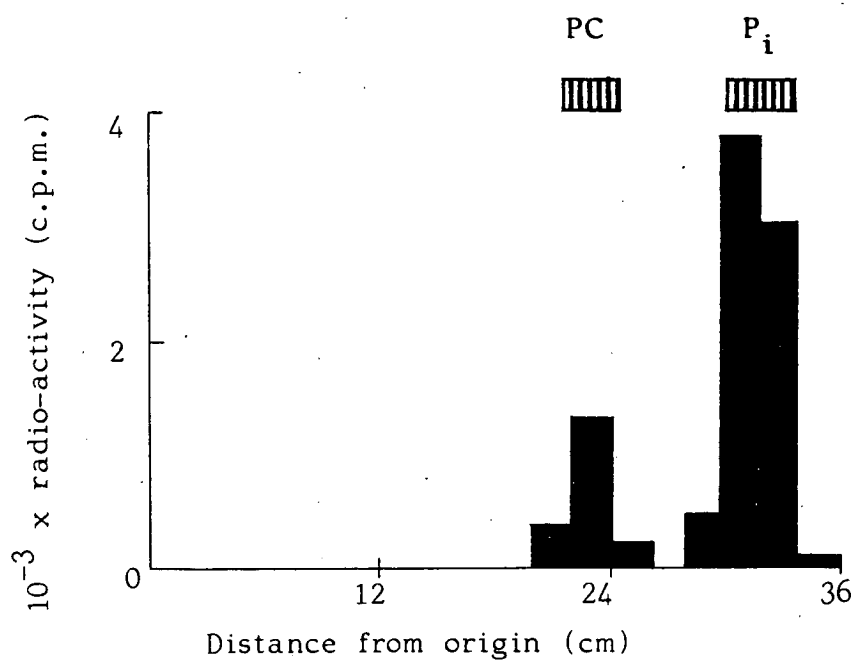
Figure 8.1 Radio-active components resolved by electrophoresis: [32 P]-PC added into rabbit kidney homogenates and a 5 min incubation period.



■ radio-activity associated with separated compounds

▨ reference standards of PC, P_i

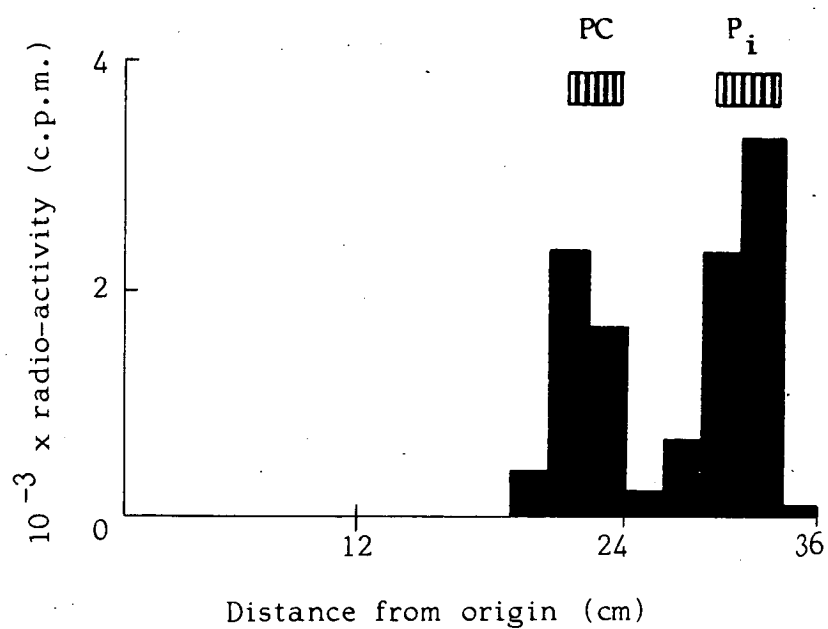
Figure 8.2 Electrophoretic resolution of [^{32}P]-labelled compounds following incubation of rabbit kidney homogenate with [^{32}P]-PC in the presence of vanadate.



■ radio-activity associated with separated compounds

▨ reference standards of PC, P_i

Figure 8.3 Effect of vanadate on [^{32}P]-PC as indicated by electrophoretic resolution of labelled compounds



■ radio-activity associated with separated compounds
▨ reference standards of PC, P_i

In other experiments, a range of inhibitors reported to inhibit alkaline phosphatase activity were tested for their inhibitory ability in kidney and intestinal homogenates. Activity at pH 7.4 and room temperature was measured using p-NPP as substrate, before and after the addition of an inhibitor. Results are given in Table 8.2 for diluted tissue homogenates. The most potent inhibitor was clearly vanadate and, significantly, some inhibitors displayed specificity. L-cysteine and L-phenylalanine were more effective in intestinal homogenates whereas tetramisole and p-bromolevamisole were particularly potent inhibitors of diluted kidney homogenates. Two inhibitors - vanadate and tetramisole - were therefore selected to investigate whether they were effective when 10% homogenates were routinely prepared. Tetramisole was selected in preference to p-bromolevamisole since its relative purchasing cost (1980: \$9.90/10g) enabled a large amount to be added into homogenates, more than compensating for its lower potency compared to its derivative, p-bromolevamisole (1980: \$42/100mg). Table 8.3 indicates that only 5mM vanadate caused > 90% inhibition. Further, at 0°C activity was $51 \pm 6\%$ that at room temperature. Unfortunately under such conditions, vanadate causes hydrolysis of PC.

Thus data indicated that hydrolytic activity in homogenates could not be inhibited sufficiently to prevent PC hydrolysis. Therefore a quantitative determination of the subcellular distribution of PC using this type of compound was not possible.

Table 8.2 Inhibition of p-NPPase activity in diluted tissue homogenates.

INHIBITOR (mM)	% INHIBITION*	
	KIDNEY	INTESTINE
EDTA (3)	67 [±] 8	68 [±] 2
l-cysteine (3)	52 [±] 5	93 [±] 1
([±])-tetramisole (3)	95 [±] 1	11 [±] 4
p-bromolevamisole (1)	96 [±] 2	14 [±] 1
thiophylline (3)	57 [±] 6	84 [±] 3
thioglycollate (3)	7 [±] 2	13 [±] 3
L-phenylalanine (3)	2 [±] 1	36 [±] 3
vanadate (1)	95 [±] 2	97 [±] 1

*3 trials; mean [±]1 S.E.

1.3g kidney was homogenised in 20ml sucrose and 50μl taken to determine the extent of inhibition by added test substances.

Table 8.3 Inhibition of p-NPPase activity in undiluted 10% tissue homogenates.

INHIBITOR (mM)	% INHIBITION* KIDNEY
Vanadate (2.5)	88 ^{±1}
Vanadate (5)	95 ^{±1}
Tetramisole (5)	61 ^{±2}
Tetramisole (10)	69 ^{±2}

*3 trials; mean [±]1 S.E.

1.3g kidney was homogenised in 15ml sucrose and 2ml taken for assay purposes.

8.3.2 SUBCELLULAR DISTRIBUTION OF PC

The question of whether PC was primarily associated with mitochondria or not was investigated by determining the PC content of mitochondria and of whole tissue. By knowing the yield of mitochondria the contribution of mitochondrial PC to the total PC content of whole tissues could be estimated. The yield was determined by monitoring succinate dehydrogenase activity.

Results are given in Figure 8.4 for a typical experiment illustrating the amount of PC initially present in the kidney and that found in mitochondria isolated from that tissue. Data show that 12 μg of PC was present in 40mg mitochondrial protein and that 48 μg of PC was present in whole kidney. The yield of mitochondria was found to be 26%, giving a corrected figure of 45 μg of PC present in the mitochondria of a whole kidney. This corresponds to 98% of the PC in whole tissue being located in mitochondria. The experiment was repeated on four separate occasions and the complete results indicate that kidney mitochondria contain 0.68nmol PC/mg mitochondrial protein and that this organelle contained 95 \pm 5% of the total PC in whole kidney.

8.3.3 BIOSYNTHESIS OF PC

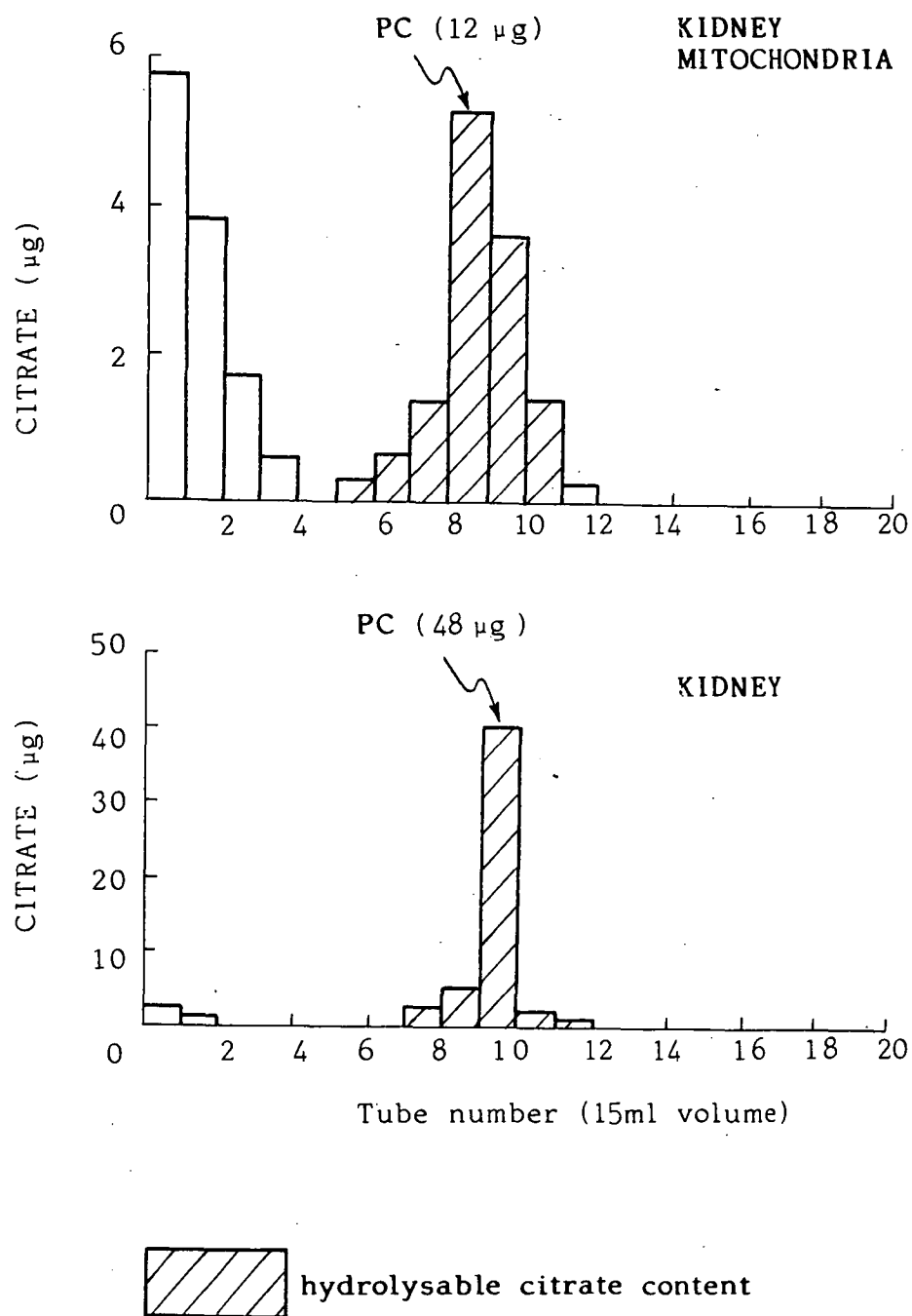
8.3.3.1 In vivo

Table 8.4 summarises data from a study in which rats were given an oral dose of calcium gluconate and were killed 30 min later. Intestinal mitochondria were isolated and analysed for PC. Notably, serum and mitochondrial

Table 8.4 Effect of an oral dosage of calcium gluconate on calcium concentrations in serum and on PC and calcium levels in intestinal mitochondria.

Levels of Ca ²⁺ or PC	Treatment	
	Ca ⁺⁺ loading	None
[Ca] mitochondria	8.6	3.9 µg/mg protein
[PC] mitochondria	0.4	0.65nmol/mg protein
[Ca] serum	20	11 mg %

Figure 8.4 Ion-exchange chromatographic separation of PC from biological extracts.



Ca^{++} levels were raised indicating the successful Ca^{++} - loading of the biological system. However, there was no evidence that PC had been synthesised under these conditions.

8.3.3.2 In vitro - Mitochondrial incubations

Rabbit kidney mitochondria were incubated in either a medium known to cause Ca^{++} -loading of the organelle, or a medium rich in citrate. Figure 8.5 summarises the results from such experiments. Mitochondria appeared to synthesize PC in response to incubation in a Ca^{++} -loading medium over 30 min; but, in these experiments, the degree of PC synthesis varied considerably. In contrast, incubation in a citrate-rich medium did not cause any PC production by mitochondria.

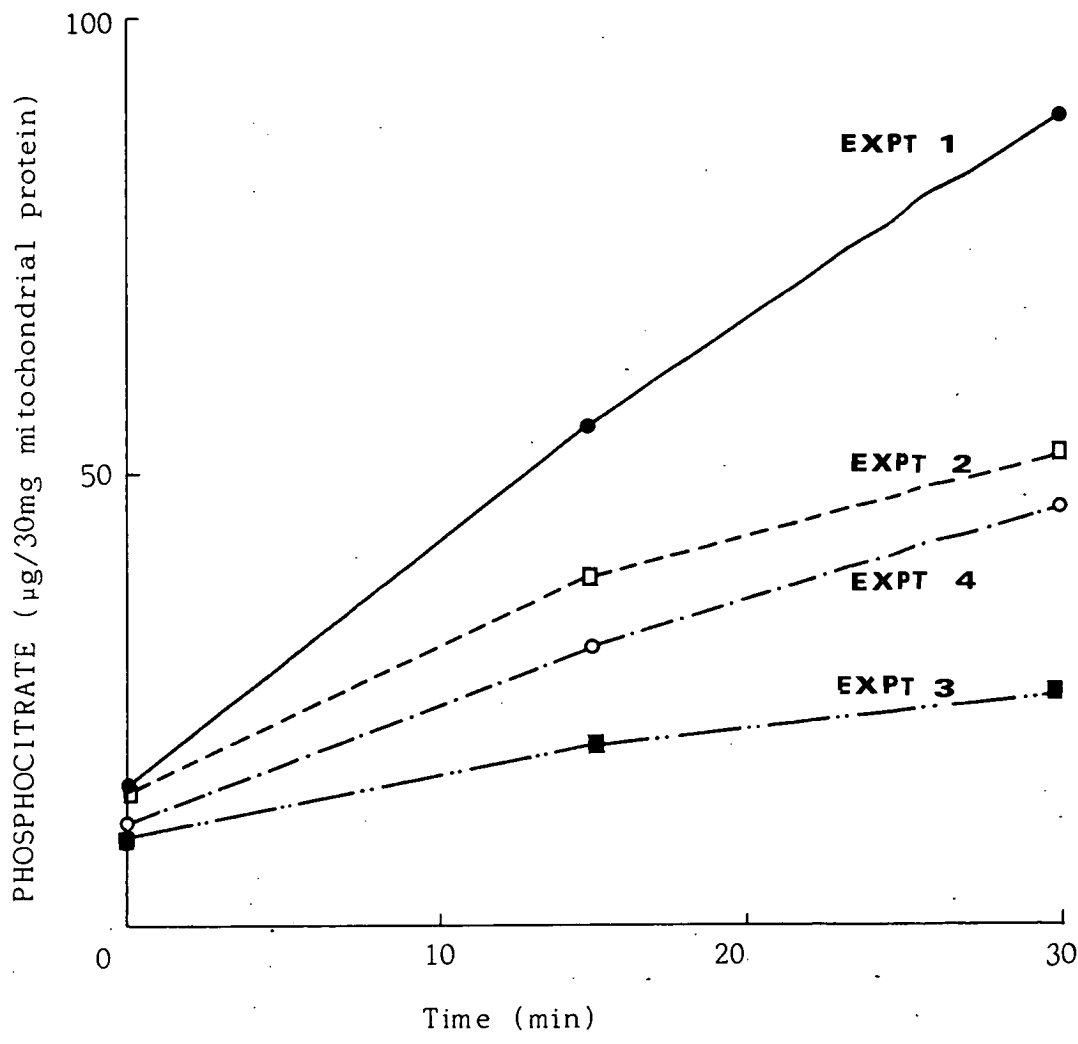
8.3.3.3 In vitro - Transphosphorylation

Attempts to prove a role for PC in transphosphorylation systems were not successful. When citrate was used in this type of reaction, no PC production was detected.

8.4 DISCUSSION

PC has been demonstrated to occur naturally, in particular associated with mitochondria. The question then arises as to whether mitochondria are the sole or major sub-cellular source of PC. One way of answering this question is to isolate cytosol and other subcellular components and to analyse them for PC. However, the in vivo lability of PC suggested that any attempt to quantitatively isolate PC from homogenates without first inhibiting the hydrolytic activity could only succeed if components were rapidly fractionated. This may explain why when cytosol from a

Figure 8.5 PC content of mitochondria incubated in a Ca^{++} -loading medium.



perfused liver was isolated by the usual differential centrifugation techniques, results (not shown here) indicated that no PC could be detected.

Although most methods for fractionating organelles involve lengthy differential centrifugation, another method for preparing cytosol is available, whereby the tissue homogenate is passed through an Affi-gel 731 column (Bio-Rad Laboratories, Richmond, California, U.S.A.) which binds membraneous components (Jacobson and Branton, 1977). Even so, this method requires at least 1h by which time endogenous cytosolic PC, if present, would be destroyed.

One recently developed differential centrifugation technique for fractionating subcellular components uses organic solvents thus apparently inactivating many enzymes, enabling metabolite concentrations in the various cellular compartments to be measured (Elbers *et al.*, 1974). However, at present this technique can only process small amounts of sample, insufficient to yield detectable amounts of PC.

Consequently, the use of inhibitors was investigated with a view to isolating cytosol by centrifugation after the addition of suitable inhibitors. A range of inhibitors was tested in rabbit kidney and intestine homogenates. Although vanadate potently inhibited tissue homogenates from both kidney and liver, tetramisole and *p*-bromolevamisole exhibited a tissue-specific effect, inhibiting only in kidney. No inhibitor was found that could entirely inhibit hydrolytic activity in tissue homogenates although 5mM vanadate approached this. However, vanadate was found to modify PC under the conditions used.

This inability then, of inhibitors to decrease markedly phosphatase activity in homogenates led to the adoption of another method to investigate the subcellular distribution of PC. In this approach, the yield of mitochondria was measured by monitoring succinate dehydrogenase activity. This enabled the mitochondrial contribution to the total PC content of whole kidney to be calculated as $95 \pm 5\%$. This important result indicates that PC is exclusively located in mitochondria.

The finding that PC is almost completely recovered from mitochondria despite the length of time required for organelle isolation, suggests mitochondrial compartmentalisation acts to protect PC from the lytic activity in the extra-mitochondrial compartment, substantiating the suggestion made in Chapter 7.

Recognition that PC is a mitochondrial metabolite suggests that this organelle is the site for the biosynthesis of PC. The proposed role of PC in stabilising calcium phosphate deposits as an amorphous rather than crystalline deposit suggests that the level of mitochondrial Ca^{++} may be a stimulus for PC production. These ideas are partly substantiated by the data presented here, but could not be supported by oral Ca^{++} -loading experiments. In these experiments, although $[\text{Ca}^{++}]$ rose in serum and in mitochondria, no increase in the intestinal mitochondrial PC levels were observed over a 30 min time period. However, when rabbit kidney mitochondria were incubated in a medium containing Ca^{++} -loading, there was a tendency to increase PC levels after 30 min, albeit erratic in the magnitude of

the increase. Substitution of 5mM citrate for Ca^{++} abolished PC production. These preliminary results, although suggesting that mitochondria do in fact synthesize PC and perhaps in response to Ca^{++} -loading, also emphasize the need for further investigations. Investigations should aim to elucidate the effect of length of incubation, source and age of mitochondria, and incubation media on mitochondrial PC production in vitro. Additional experiments could establish the way in which the nutritional status of rats might affect the PC content of mitochondria in different tissues. The high and low calcium diets used by Omdahl and DeLuca (1973) might be particularly useful in this regard.

Elucidation of the optimal conditions for PC biosynthesis could prove useful for preparing [^{32}P]-PC biologically as an alternative to its chemical synthesis. In fact the use of [^{32}P]- P_i in biological experiments will be desirable since ion-exchange chromatography followed by high-voltage electrophoresis, as described in the last chapter, should allow for an accurate determination of the types of phosphoric esters present and the extent of their biosynthesis. The investigation of the transphosphorylation of phosphatases would also be aided by the use of [^{32}P]- P_i . Preliminary results presented here indicate PC cannot be produced by transphosphorylation, but other enzymes and conditions need to be tested to reinforce this conclusion. This requirement is emphasized by the following observations:

1. Glycerol has been phosphorylated by intestinal alkaline phosphatase (Kay, 1928), albeit in a reaction medium with high concentrations of glycerol and phosphate.

At the bone site there is a high concentration of both phosphate and citrate (up to 2% the dry weight of bone [Dickens, 1941]) so that conditions here are favourable for PC production by reverse phosphatase activity. There this apparently artificial reaction could have physiological significance.

2. Different phosphatases show different substrate preferences. For example, intestinal alkaline phosphatase phosphorylates compounds in order of preference: ethanolamine > tris > glycerol >> lactose, L-serine = 0 (Linden and Alais, 1976), whereas a glycoprotein from pre-osseous cartilage phosphorylates in the order L-serine >> tris > diethanolamine (Stagni et al., 1979).

Preliminary data suggests Ca^{++} -loading may stimulate mitochondrial PC production, at least in vitro; the PC content in tissue (i.e. liver << kidney < intestine) may therefore be related to levels of Ca^{++} present in these tissues and to the flux of Ca^{++} at these sites. In this model the presence of PC in periods of Ca^{++} -loading is needed to ensure amorphous rather than crystalline calcium phosphate is deposited in the mitochondrial matrix. The solubility and lability of ACP relative to HAP ensures Ca^{++} can be readily mobilised from the mitochondrial Ca^{++} 'sink'. This reasoning highlights the need to investigate whether or not chondrocyte mitochondria contain PC and, in particular, the potential role of PC in biomineralisation since it has been shown that prior to mineralisation, mitochondrial calcium phosphate granules disappear and simultaneous extracellular calcium phosphate deposits occur.

The data presented in this chapter indicate PC in a mitochondrial metabolite and tentatively suggests it is synthesized by this organelle in response to Ca^{++} -loading. The information also indicates future avenues of research worth investigating.

8.5 SUMMARY

1. PC was found to be rapidly hydrolysed in rabbit kidney homogenates.
2. Kidney and intestine phosphatase activity was inhibited by a range of inhibitors: vanadate, tetramisole, L-cysteine and L-phenylalanine. Only vanadate approached complete inhibition; but vanadate caused breakdown of PC.
3. Mitochondria from rabbit kidney were found to contain $95 \pm 5\%$ of the total PC found in that tissue.
4. Ca^{++} administered orally did not cause mitochondrial PC production after 30 min.
5. Rabbit kidney mitochondria incubated in a Ca^{++} -loading medium produced PC. Substitution of citrate for Ca^{++} in the medium prevented PC production.
6. PC was not produced by transphosphorylation of citrate with phosphocreatine mediated by calf intestine alkaline phosphatase.

CHAPTER 9

GENERAL DISCUSSION^{*}

Calcium homeostasis involves control at many levels including the subcellular level (for example, Ca^{++} flux in mitochondria), the cellular level (for example, ATP energised uptake and release of Ca^{++}), and at the organ level mediated by hormones (for example, parathyroid hormone and vitamin D). However, the useful operation of these regulatory mechanisms demands that the formation and resorption of Ca^{++} salts from both intracellular (for example, mitochondrial) and extracellular deposits (for example, bone) must be tightly controlled. Although investigations into the role of Ca^{++} and P_i concentrations, and nucleating agents on Ca^{++} salt precipitation have been published, much interest has recently been focussed on the role that inhibitors of this process might play in regulating Ca^{++} salt formation. Many compounds have been considered as inhibitory factors influencing biological calcification. For example, pyrophosphate has been suggested as a compound preventing lithiasis in normal individuals (Fleisch and Bisaz, 1964; Fleisch and Russell, 1970), and/or causing bone mineralisation through its removal by matrix vesicles (Felix and Fleisch, 1976). Another compound suggested in this latter role is ATP but as with pyrophosphate many factors - such as the ubiquity of these organic phosphate esters - have led to these inhibitors not being generally accepted as controlling calcification (Posner, 1969; Posner et al., 1978). Nevertheless, the role that inhibitors

^{*} Some of the work presented here has been published by the candidate and his supervisors - copies are enclosed in the thesis. See statement in Acknowledgements.

could play in influencing biological calcification is considered so important that much current research aims at finding new inhibitory factors that might control Ca^{++} salt deposition in biological systems.

At the commencement of this present investigation, current reports indicated that a fraction potentially inhibiting HAP formation could be isolated from such diverse sources as milk, mitochondria and urine (Howard, 1976; Tew and Mahle, 1977; Lehninger, 1977; Lehninger et al., 1978). However, the identity of the agent(s) responsible for inhibition was unclear because little evidence existed enabling the elucidation of its structure. From unpublished ^1H NMR data, Howard (1976) reported that the material contained a citrate molecule and, in another approach, found that by acidifying and neutralising solutions of P_i and citrate in vitro, a potent inhibitor was formed. Because this artificial compound appeared to be chromatographically similar to the natural inhibitor, it was proposed that the inhibitor might be phosphocitrate(PC).

The natural occurrence of this inhibitory compound was thrown into doubt by the observation that ion-exchange chromatography under acidic conditions - the method used to purify biological fractions - caused the production of the inhibitory agent from P_i and citrate (Howard, 1976). Another limitation of these studies was the fact that no authenticated PC was available to enable a detailed comparison with biological components, nor could the ability of pure PC to inhibit HAP be unambiguously determined. Hence Lehninger (1977) cautioned that the active component in the biological fraction might not be PC but some other

compound isolated with it and added that "rigorous identification will require an unambiguous high-yield organic synthesis of PC and comparison with the highly purified naturally-occurring inhibitor".

Consequently the studies reported in this thesis initially aimed at preparing and unambiguously characterising PC. A further requirement was that the organic synthesis should allow isotopic labelling of PC, since such a compound would be of great value in biological experiments. The availability of PC was also seen as allowing the development of methods for isolating and analysing PC which could ultimately be applied to biological samples to prove definitively the presence or absence of PC in nature.

Meyer et al. (1959) had, in fact, originally reported a synthesis of PC but it was a low-yield method, used specialised equipment, and required reagents that did not facilitate isotopic labelling of PC. A more powerful phosphorylating reagent was indicated and later Tener (1961) described the use of cyanoethylphosphate (CEP) to phosphorylate nucleosides. This reagent not only was more powerful than the reagent employed by Meyer et al. (1959) - dibenzyl chlorophosphate - but also permitted [^{32}P]-labelling because of the easy preparation of [^{32}P]-CEP from [^{32}P]- P_i .

Recently a new potent phosphorylating reagent - o-phenylene chlorophosphate - has been reported (Khwaja et al., 1970) that reacts even with tertiary, sterically hindered hydroxyl groups which is the type that must be phosphorylated to prepare PC. However, using o-phenylene chlorophosphate, isotopic labelling of PC is difficult since either

[^{32}P]- PCl_5 must be used to prepare the phosphorylating reagent by a lengthy synthetic method, or [^{14}C]-citrate must be esterified to give triethyl citrate, one of the required starting materials. Nevertheless, because of the potential high yields available using *o*-phenylene chlorophosphate, its use in preparing PC was investigated along with CEP, the reagent foreseen as enabling ready isotopic labelling of PC.

Condensation of CEP with triethylcitrate (TEC) mediated by dicyclohexyl carbodi-imide (DCC) followed by alkaline hydrolysis gave crude PC. The development of an ion-exchange system using bicarbonate buffers and operating at neutral pH enabled the purification of PC. With nitrate as the eluting ion, even better resolution of P_i , citrate and PC was obtained; but with bicarbonate buffer all that is needed to recover PC is to decarbonate the buffer with AG 50W [H^+], whereas with nitrate, insoluble salts must be formed. Consequently, ion-exchange chromatography using sodium bicarbonate buffers was used to give sodium PC which was characterised by a range of techniques. NMR spectroscopic data was used to provide unambiguous proof of the structure of the prepared compound. Not only ^1H NMR spectroscopy was used for this purpose, but also ^{31}P and ^{13}C NMR spectroscopy, the latter giving a unique spectrum characterising PC completely. Further characterisation was obtained using thin-layer chromatographic, chemical and enzymatic systems, especially developed for separating and analysing PC (Chapter 2; Williams and Sallis, 1980; Williams and Sallis, 1981a).

The overall yield obtained using CEP was generally poor (ca. 10%); but using o-phenylene chlorophosphate a higher yield appeared possible since the initial phosphorylation of triethyl citrate was almost quantitative. The removal of a protecting group, however, by either oxidative or reductive cleavage, proved troublesome. Oxidative cleavage was found to produce a dye which not only formed a polymeric material but bound non-specifically to ion-exchange resins disrupting binding and resolution. The selective removal of the dye was not successful using a range of adsorbents including activated charcoal, Florisil and Celite.

The other alternative, reductive cleavage, requires platinum oxide (an expensive reagent) resulting in the formation of 1,2-cyclohexyldiol in contrast to a quinone produced if oxidation is used. This diol should not interfere with ion-exchange techniques. The use of reductive cleavage was not investigated because it was found that complete hydrogenation of the intermediate was difficult to achieve, yet was necessary to avoid the formation of dye. In any case isotopic labelling was easier and produced a higher specific activity product if [^{32}P]-CEP was used. Although [^{14}C]-citrate could have been used to prepare [^{14}C]-triethyl citrate for reaction with o-phenylene chlorophosphate, the low specific activity of commercially available [^{14}C]-citrate compared with [^{32}P]- P_i which is also significantly less expensive, led to the use of P-32 as the isotopic label for PC. Despite the shorter half-life of P-32 compared with C-14, the higher specific activity product obtainable was adjudged to be of particular advantage for experiments where

the fate of labelled PC administered to rats was to be determined. Therefore the CEP method is the method-of-choice for preparing isotopically labelled PC.

Tew et al. (1980) reported a synthesis of PC using o-phenylene chlorophosphate just after its preparation from CEP had been published (Williams and Sallis, 1980), which confirmed some of the data reported here. A yield of 30-80% was reported by Tew et al. (1980), which is higher than that available using CEP (ca. 10%). Consequently, as expected due to the reactivity of o-phenylene chlorophosphate, this reagent is superior for the preparation of unlabelled PC. Nevertheless, several improvements to the method reported by Tew et al. (1980) can now be suggested based on the findings using CEP.

For example, hydrolysis in the presence of Ca^{++} could be improved since Meyer et al. (1959) noted that the mono-ethyl ester of PC was quite soluble - even in the presence of Ca^{++} . This may explain the variable yield obtained by Tew et al. (1980) who used a short hydrolysis time and insufficient excess of Ca^{++} . PC is quite stable at 0°C and is precipitated using excess Ca^{++} so a useful modification would be to extend the hydrolysis time from 4h to 16h and to use excess Ca^{++} , in amounts described here (see Chapter 2). The more efficient removal of ethyl ester groups combined with the precipitation of PC should optimise the yield of this method.

An additional improvement would appear to be the substitution of the AG 1-X8 (HCO_3) system developed here

for the cellulose-based system used by Tew et al. (1980) which necessitates formation of the undesirable Ca^{++} salt. In contrast, the ion-exchange system enables clear resolution of P_i , citrate and PC, has a higher binding capacity and, further, allows the sodium rather than the Ca^{++} salt to be routinely prepared. Clearly for studies of calcification it is desirable to have more soluble sodium salt, uncontaminated with divalent metal ions.

The method of Tew et al. (1980), using o-phenylene chlorophosphate, improved by altering the hydrolysis conditions and substituting the ion-exchange method developed for the CEP synthesis should provide an optimal synthesis of PC. The CEP method, however, appears better for producing isotopically labelled PC since [^{32}P]-PC yielded with a high specific activity.

Both syntheses satisfy the criterion reported by Lehninger (1977) noted above, but even with the availability of authentic material, resolution of biological components and methods for identifying PC in biological extracts required the development of methods for the separation and analysis of PC which did not previously exist. Although NMR and mass spectroscopy provide information on the structural nature of a compound, with biological samples it is not always possible to obtain sufficient amounts of pure material for analysis by these spectroscopic methods. Instead, a range of techniques for characterising PC were established - chemical and enzymatic assay, TLC, high-voltage electrophoresis and analytic isotachophoresis - more valuable than any single method since each method has some limitations

(Chapter 3; Williams and Sallis, 1981a).

Chemically, citric acid can be colorimetrically determined after degradation to pentabromoacetone (PBA) and reaction with benzidine (Jones, 1967). PC can be estimated by this method if prior hydrolysis is carried out. An alternative to benzidine is 4,4'-dimethylaniline; it was found that, although it did not give as intense a colour reaction as benzidine, it did give a linear response to citrate over a wider concentration range. The use of both reagents therefore constitutes a flexible citrate assay. Unfortunately PC itself gives some reaction in the assay system, presumably due to partial hydrolysis, so this method can only be used to measure total citrate.

A method to determine free citrate and PC, however, was developed based on the sensitive and selective enzymatic citrate assay of Passonneau and Brown (1974). One portion of a sample was analysed for citrate, and another was treated with alkaline phosphatase to cleave PC. Free citrate was measurable since it was found that citrate lyase did not degrade PC to citrate. Total citrate (citrate + PC) was determined after phosphatase activity had selectively cleaved PC into phosphate and citrate. This fluorimetric assay system is particularly valuable because of its sensitivity (0.05-10 μg) and ability to selectively analyse citrate and PC.

A thin-layer chromatographic system was also developed that separated citrate and P_i from PC, although citrate and P_i were themselves not clearly separated from one another. Citrate and P_i , however, could be selectively visualised

due to the range of staining techniques available (Chapters 2, 3). PC was detectable after either chemical or enzymatic hydrolysis.

Another separation technique for use in resolving PC from other compounds was high-voltage electrophoresis. The procedure of Khwaja et al. (1970) was applied successfully to the resolution of PC and P_i although citrate and PC were not so definitively separated. Consequently this method was foreseen as of potential advantage in separating [^{32}P]-labelled components.

Analytical isotachophoresis was another technique applied to the resolution of PC from other anions. Anions separate into discrete zones using this method, each with a unique conductivity which serves to characterise them when conductimetric methods are used to detect resolved components. Although this method is of great potential use, one major disadvantage is that if salts are present in sufficient concentration then clear-cut resolution is lost.

A preliminary purification is required before any of these separation or analytical techniques can be used because (1) of the high concentration of citrate relative to PC present in most of the biological extracts, (2) a need to separate salts from material to be analysed, and (3) a necessity to remove fluorometric species interfering with enzymatic analyses. Ion-exchange chromatography was found to be suited to this purpose since it has a high-binding capacity and it was not found to cause PC production from P_i and citrate as an artefact of chromatography. Furthermore,

if chromatography was omitted and a solution of citrate and P_i (in concentrations ca. 100 times that present in biological extracts) subjected to the acidification and neutralisation methods used to prepare biological extracts, again PC was not produced. Therefore biological extracts were prepared and purified by ion-exchange chromatography with the knowledge that if PC was detected it was derived from the sample and not artefactually. The "PC-rich" fraction was then analysed by a range of techniques.

Whereas a range of separation systems - TLC, electrophoresis and analytical isotachopheresis - was found to be useful in characterising PC, GLC could not be applied. Derivatisation by methylation with thionyl chloride failed, as did trimethylsilylation using a wide variety of reagents and conditions. However, only in one instance were P_i and citrate detected after trimethylsilylation of PC suggesting that either no derivatisation of PC was occurring or complex fragmentation resulted from the required conditions. Diazomethane is the only other reagent which is powerful enough yet could react under relatively mild conditions that might esterify PC enabling GLC. In fact, Tew et al. (1980) have used diazomethane to derivatise PC but reported that only the trimethyl-ester rather than the necessary penta-methyl ester, was obtainable. A wide range of derivatisation reagents and reaction conditions are therefore apparently not applicable to PC.

It is not surprising then that Sutor et al. (1979a) reported there was no evidence for the existence of PC in urine, using GLC techniques. This finding, together with

the observations that artificial urine inhibited the transformation of ACP into HAP to the same degree as natural urine, led Sutor *et al.* (1979b) to propose that all inhibitors in urine are known. This conclusion, however, is questionable, since the artificial urine used was oversimplified; it did not contain components found in urine known to promote Ca^{++} salt precipitation (Hallson and Rose, 1979; Fleisch, 1978) which would lead to an underestimation of the inhibitory capacity of urine. Consequently, it is argued here that a role of PC in the prevention of urolithiasis is still an open question.

If future experiments confirm the preliminary results here that indicate PC is found in urine, then several interesting questions arise, including: (1) does PC concentration fall in patients predisposed to form stones? (2) is its concentration higher in tubules than when voided? (3) does it possess the physico-chemical properties enabling it to inhibit lithiasis? Although the first question cannot be answered at present, speculation can be made on the other points. The stability of PC in urine (Chapter 7) suggests its concentration at the time of voiding is the same as at its time of secretion. However, it may be that the epithelial lining at the proposed site of secretion may possess some hydrolytic activity so that PC after acting to inhibit stone nucleation in the kidney tubules is rapidly degraded. Clearly further work is necessary to clarify these points.

If PC is to play a role in urolithiasis, or indeed any calcifying system, it must have the ability to inhibit calcification. Synthetic PC has been tested *in vitro* for its

ability to inhibit calcium oxalate and hydroxyapatite (HAP) precipitation. The results presented in this thesis show PC to be the most potent of a range of powerful inhibitors of HAP formation including ATP, EHDP, PNP and PP_i (Chapter 4; Williams and Sallis, 1979, 1981b; Williams *et al.*, 1980). Although Tew *et al.* (1978) reported that the "purified factor" - presumably PC - could not inhibit without the presence of citrate, these workers have recently published reports which confirm the findings presented in this thesis (Tew *et al.*, 1980; Reddi *et al.*, 1980). These observations strengthen the possibility that it is indeed PC which is responsible for the inhibitory ability of certain biological fractions.

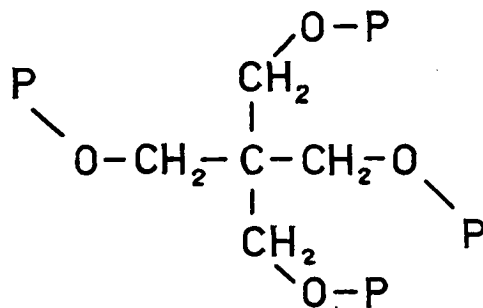
With regard to calcium oxalate formation, PC is, weight-for-weight, more potent than chondroitin sulphate C (Chapter 4; Williams and Sallis, 1981a) one of the most potent inhibitors of calcium oxalate formation. Although on a molar basis chondroitin sulphate C is more potent than PC, PC is more potent on this basis than pyrophosphate, a moderately powerful inhibitor. Recently Tew and Malis (1981) have demonstrated that PC inhibits the epitaxial growth of calcium oxalate upon HAP. These observations indicate that if PC is found in sufficient quantity in urine, then it has the physico-chemical properties to make it an important factor influencing urolithiasis.

The unique structure of PC has led to an understanding of how the structure of a molecule influences its ability to inhibit HAP formation (Chapter 5; Williams and Sallis, 1979). It is proposed here that for potent inhibition to occur a molecule should possess at least one phosphate group and

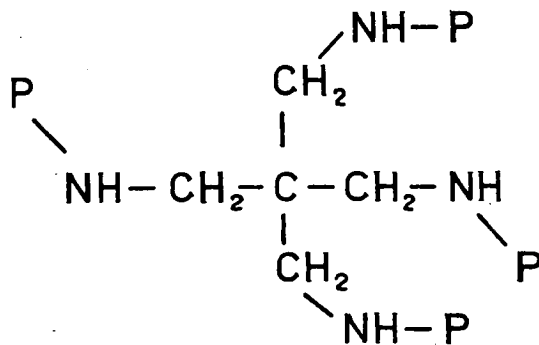
- at some other position - either a phosphate group preferably or a carboxylic moiety. A wide range of inhibitors has been tested, and this has led to an extension of this basic rule to include the effect of secondary factors on inhibitor strength (Chapter 5; Williams and Sallis, 1981b). These are (i) the number of active groups, (ii) their proximity, (iii) stereochemical arrangement, (iv) steric factors, (v) lipophilicity, and (vi) lability of the molecule. It was also noted that primary amino groups abolish inhibitor potential presumably by altering binding characteristics.

This information should not only enable the recognition of biological compounds previously overlooked as inhibitors, but should also permit the development of potent anticalcifying compounds potentially useful in the therapy of Ca^{++} metabolism disorders. Three compounds of interest optimally satisfying the structural criteria mentioned are shown in Figure 9.1. All have four phosphate groups radiating from a central carbon. However, the bridge between phosphorous and carbon is different in these compounds: P-O-C, P-N-C, P-C-C. Results presented in this thesis on the lability of PC in vivo, and published data on the in vivo stability of pyrophosphate indicates compound I is to be expected to be rapidly degraded by enzymatic hydrolysis. Therefore compounds II and III with stabler P-X-C (X may be C or N) bonds are predicted to be of more clinical value. Furthermore, since these compounds are predicted to be more potent inhibitors than PC they may ultimately prove to be more useful in therapy than PC or its analogues. Synthesis of compounds I and II from pentaerythritol is envisaged.

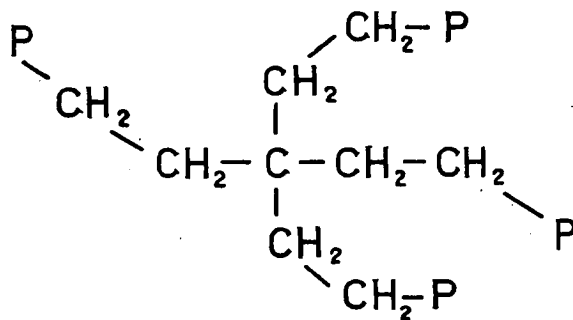
Figure 9.1 Derivatives of pentaerythritol predicted to be potent inhibitors of HAP formation.



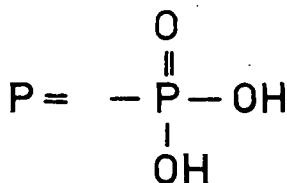
COMPOUND I = 'tetraphosphopentaerythritol'



COMPOUND II = N-analogue of compound I



COMPOUND II = C-analogue of compound I



Another application of this structure-activity relationship is to the elucidation of the mechanisms of inhibition at the calcium phosphate surfaces. Such an application has been facilitated by the recognition that inhibitors group into two classes depending on how their increasing concentration affects parameters defining the transition of ACP into HAP (Chapter 5; Williams and Sallis, 1981b). One group - Type I inhibitors - that includes PC, are crystal-growth inhibitors which act by binding to active growth sites on HAP. The other group - Type II inhibitors - apparently act at the ACP surface to decrease its lability, and do so by a variety of mechanisms. Type II inhibitors are a diverse range of inhibitors including Mg^{++} , citrate and ATP. Type I inhibitors, in contrast, appear to share some common structural features. They are low molecular weight compounds containing one phosphate group and in close proximity, either another phosphate group or multiple carboxyl groups, but no bulky inert groups (for example, an adenosine moiety).

Another interesting observation to arise from the recognition of these two classes of inhibitors is the synergistic effect two inhibitors, comprising one inhibitor from each group, has on HAP formation. This probably arises from an efficient binding to different active sites on HAP. In particular, PC acts synergistically with phosphoglycerate and citrate. Mg^{++} requires special consideration since its action is probably to protect phosphoric esters from hydrolysis on calcium phosphate surfaces, through its chelating ability (Blumenthal et al., 1977). PC also acts synergistically with Mg^{++} , so the physiological situation can be visualised

where PC acts as a potentiating agent, a small amount sufficient to dramatically increase the inhibitory ability of a physiological fluid. No such synergism was found between ions in the inhibition of calcium oxalate formation.

The recognition of these two groups of inhibitors and mechanisms of inhibition does not preclude the possibility that other mechanisms and types of inhibitor exist. For example, proteoglycans do not significantly affect the induction time but alter the rate and extent of transformation (Blumenthal et al., 1979). One explanation might be that the molecule binds to the ACP spherules and sterically hinders the surface-dependent processes necessary to transform ACP into HAP. Whatever the exact cause this finding emphasises that high molecular weight inhibitors may inhibit by mechanisms other than those discussed here.

The wide range of compounds that can inhibit the formation of HAP casts doubt on the claim by Tew et al. (1978) that "using assays which measure the rate and extent of transformation of ACP into HAP and the rate of HAP crystal growth, we have found mitochondria contain phosphocitrate". Nevertheless, this fortuitous insight has now been proved to be correct by the more rigorous approach used in this study. The subsequent examination of biological fractions was made possible through the establishment of systems for the isolation, detection and analysis of PC (Chapter 6; Williams and Sallis, 1981a).

Rat and rabbit liver and kidney homogenates examined for PC content were found to give, after ion-exchange chromatography, a fraction which, when hydrolysed, contained

P_i and citrate. Furthermore, the compound eluted with the same elution volume as authentic PC. Enzymatic analysis provided additional evidence that the compound isolated was PC for only when the compound was incubated with a phosphatase was it possible to obtain a reaction with citrate lyase. Comparison of the biological fraction with authentic PC by thin-layer chromatography, high-voltage electrophoresis and analytical isotachophoresis confirmed its identity. However, results with bone and urine are not unequivocal since their high concentrations of citrate, P_i and other anions made purification difficult and only small amounts of PC were detectable. Hence, although a qualitative indication of the presence of PC was obtained in both cases, a more detailed examination is indicated.

This demonstration that PC exists in nature led to an examination of the suggestion that mitochondria might be an important source of PC (Howard, 1976; Lehninger, 1977). Mitochondria from rabbit liver, kidney and intestine were found to contain PC and on preliminary estimates contained 0.4, 0.8 and 1.2mmol PC/mg mitochondrial protein, respectively, although the intestinal source requires further study before the exact PC level can be confidently ascertained (Chapter 6, Williams and Sallis, 1981a, c). It is likely that PC levels are influenced by the status of the mitochondria, or indeed the whole animal, with respect to Ca^{++} and P_i levels.

The increasing order of PC levels in these mitochondria correlates with an increasing Ca^{++} flux through the tissue, perhaps reflecting a need to maintain calcium phosphate

deposits in the amorphous phase which is relatively labile (Termine and Eanes, 1972), enabling rapid mobilisation of Ca^{++} . This reasoning emphasises the desirability of a study to analyse chondrocyte mitochondria for PC, since large Ca^{++} fluxes occur at the mineralisation site.

In order to gain data that might assist in the definition of the biological roles of PC, [^{32}P]-PC was given intravenously to rats and the tissue distribution and lability of [^{32}P]-PC determined. Comparative experiments with [^{32}P]- P_i aided interpretation of results, and specific points arising from such a study (Chapter 7; Williams and Sallis, 1981a, d) include the following: (1) major sites of PC uptake were bone, consistent with its in vitro properties, and kidney; (2) PC is not metabolised directly but first hydrolysed to citrate and P_i ; (3) in contrast to the stability of PC in blood, the compound is very labile at the kidney site. This latter finding suggests: (a) special conditions prevail at the kidney site for the hydrolysis of PC; (b) compartmentalisation must act to protect PC from the lytic activity in the extramitochondrial compartment since PC is detectable in kidney mitochondria; (c) PC may be of use itself in preventing arterial calcification since it is stable in blood. However, for the treatment of nephro- and urolithiasis PC is not likely to be very suitable. Although PC is stable in urine, passage from blood into kidney then urine results in extensive hydrolysis of the compound emphasising the need for stable analogues.

At the time these results on the in vivo lability of PC were published (Williams and Sallis, 1981a), Tew et al.

(1981) reported work in which an injection of PC prior to administration of parathyroid hormone led to an inhibition of subsequent nephrolithiasis. Significantly, however, this total effect is not likely to be due to PC alone since it is rapidly hydrolysed at the kidney site. Tew et al. (1981), who concluded PC might have some clinical usefulness in the treatment of lithiasis, were unaware of the degree of lability of PC in vivo.

A possible explanation for the apparent inhibitory action of PC is that PC is selectively taken up by the kidney - the second most important site for PC uptake - where it is rapidly hydrolysed to P_i and citrate which by virtue of its weak anti-calcifying ability inhibits nephrolithiasis. In this model PC acts to concentrate citrate - a weak inhibitor - in sufficient amounts in the kidney to cause inhibition. If indeed this is the mechanism, then administration of citrate should be as effective as PC, provided kidney uptake is similar.

After the presentation of results indicating the in vivo lability of PC (Williams and Sallis, 1981a), in which it was suggested that an analogue of PC could be of more therapeutic use than PC, Reddi et al. (1980) reported an experiment where PC was found to be unable to inhibit ectopic calcification. In this study, demineralised bone matrix implants were used to induce calcification and the inability of PC to inhibit was suggested to be due to in vivo hydrolysis, although no direct evidence substantiating this claim was presented. This explanation would be in keeping with the now known lability of PC, especially when the phosphatase activity,

which is rich in mineralising bone, is taken into account. Reddi *et al.* (1980) also concluded that an analogue of PC stable to in vivo enzymatic hydrolysis is necessary for biological experiments, confirming previous suggestions (Williams and Sallis, 1981a).

With consensus on the desirability of an analogue of PC for use in biological systems, the question arises as to which of the compounds would be of most potential use. The analogues of PC with a P-C-C bond would be predicted to be most inert in biological systems and therein lies its potential disadvantage. Diphosphonates, inert analogues of pyrophosphate, have undesirable long-term effects because they bind to the skeleton causing an irreversible decrease in bone turnover (Bone *et al.*, 1979; Fast *et al.*, 1977, 1978) and adversely affect vitamin D metabolism (Parry and Avioli, 1981). For this reason, PC itself and its analogue with a P-N-C bond may be more useful because of their in vivo lability. PC, which is stable in blood, is of potential use in preventing arterial calcification and as an agent decreasing bone turnover which could eventually be destroyed. Reduction in bone turnover is necessary for treating Paget's disease for example, and also bone lysis due to sarcoma. The P-N-C analogue may also prove useful for reducing bone turnover, but its particular advantage is due to its potential stability in the kidney. If, in contrast to PC, the P-N-C analogue is stable in the kidney, and if throughput into the urine is sufficient, then the possibility arises of preventing lithiasis. A list of some of the potential clinical applications of phosphocitrate and its analogues is given in Table 9.1.

Table 9.1 Some potential clinical uses of phosphocitrate or its analogues*

Ecotopic calcification

Myositis ossificans progressiva

Myositis ossificans of other types

e.g. paraplegia, hip surgery.....

Calcinosis of various types

e.g. scleroderma, tumeral calcinosis

Vascular calcification

Dental calculus and caries

Excess bone turnover

Paget disease

Bone dysplasias

Excess bone loss

Acute osteoporosis

e.g. immobilization, paraplegia

Post menopausal and senile osteoporosis

Chronic renal failure

Inoperable parathyroid adenoma and parathyroid carcinoma

Bone metastases, myeloma

Rheumatoid arthritis

Ankylosing spondylitis

Calcium-containing renal stones

Bone-scanning agents

e.g. $^{99m}\text{Tc-Sn-EHDP}$

*Based on the possible clinical applications of diphosphonates given by Russell *et al.* (1981).

The lability of PC at the kidney site indicates that since endogenous PC is detectable in kidney mitochondria, then compartmentalisation within this organelle protects PC from hydrolysis. In an attempt to confirm this conclusion and to study the subcellular distribution of PC, the contribution of mitochondrial PC to the total PC content of whole kidney was measured. It was found that rabbit kidney mitochondria contain $95 \pm 5\%$ of the total PC found in this organ. This result indicates that PC is a mitochondrial metabolite and suggests that its biological role(s) is/are based on its action in mitochondria. The information supports the speculative claim that PC acts to stabilise the calcium phosphate granules that form in mitochondria in the amorphous form.

With PC established as a uniquely mitochondrial metabolite, the problem of its biosynthesis was studied, especially with regard to this organelle being the likely site of PC production. Rats given an oral load of Ca^{++} , however, failed to show an increase in PC levels in intestinal mitochondria, despite the demonstration that the Ca^{++} level in mitochondria rose. This approach - Ca^{++} -loading - was continued since Ca^{++} flux, or more explicitly Ca^{++} -loading, and deposition of its phosphate salt in mitochondria, is the process in which PC is considered to act. In fact, kidney mitochondria incubated in a medium which is known to cause massive Ca^{++} -loading, did show an increase in PC levels, albeit an irregular one. The substitution of citrate for Ca^{++} did not cause an increase in PC levels.

These data support the proposed biological role for

PC, but further experimentation to determine the exact stimuli for PC production is required. The enzyme(s) responsible for PC biosynthesis in mitochondria is/are unknown at this stage. Attempts to phosphorylate citrate by a transphosphorylation using calf intestine alkaline phosphatase failed, but a more thorough investigation of this possible method for PC biosynthesis is indicated. Different phosphate donors, incubation medium and alkaline phosphatases need consideration.

The mitochondrial location of PC and its biosynthesis under Ca^{++} -loading conditions is the closest that evidence has come to proving absolutely that PC acts in mitochondria to ensure HAP does not crystallise. Although such a generalised role can be envisaged to occur in all mitochondria, this basic role must be modified in specific tissues. Already it has been shown that PC levels are different in mitochondria from various tissue sources correlating with levels of Ca^{++} flux expected at those sites.

At the bone site possible roles for PC need special consideration. It has been suggested that mitochondria are intimately involved in the mineralisation process. Recent EM evidence (Matthews et al., 1970; Brighton and Hunt, 1978a, b) indicates that the calcium phosphate amorphous granules form in the mitochondria prior to calcification of the epiphyseal plate. Such deposits disappear simultaneously with the appearance of HAP needles in matrix vesicles - small, phosphatase-rich bodies found at the mineralisation front. The detailed molecular mechanism responsible for this transformation into HAP is unknown but PC can be envisaged to play a role in this process since it is labile to

phosphatase activity. Consequently, PC could act to stabilise mitochondrial deposits in the amorphous phase; but once deposits are transferred to matrix vesicles, phosphatases inactivate PC allowing the transformation of ACP into HAP needles to proceed.

There is, however, another important action PC may have at the bone site. The Robison hypothesis of bone formation (Robison, 1923) states that phosphatase activity at the calcifying site hydrolyses organic phosphoric esters elevating local P_i concentrations and providing the impetus for calcification. This idea has been the focus of much research interest since its initial proposal in 1923, but because of (1) the ubiquity of alkaline phosphatase, and (2) the lack of an organic phosphate unique to the calcifying site, the hypothesis has been unable to explain satisfactorily why calcification only occurs at certain well-defined sites (Posner, 1969). Examples of phosphoric esters whose ubiquity has made models of bone formation involving their hydrolysis contentious, are ATP (Leonard and Scullin, 1969) and pyrophosphate (Fleisch and Russell, 1970).

In contrast to models based on ATP or pyrophosphate, any proposed model involving PC as the agent responsible for increasing phosphate concentrations can account for the specificity of mineralisation. It is possible that PC could be delivered to the calcifying site in a controlled fashion since it is associated with mitochondria. Once at the site of mineralisation, its hydrolysis would boost the local P_i concentration. Thus PC could have a dual role: unhydrolysed it acts to inhibit HAP precipitation, whereas after

hydrolysis, one of its cleavage products promotes hydroxy-apatite formation.

Evidence that phosphatase activity influences mineralisation is, in fact, available. Alkaline phosphatase levels are known to rise markedly just before calcification (Bernard, 1978; Firschein and Urist, 1971; Althoff et al., 1978). Further, a recent report has indicated that if phosphatase activity is inhibited by L-tetramisole (levamisole) then mineralisation does not proceed (Fallon et al., 1979). Notably the dextro isomer of tetramisole does not inhibit alkaline phosphatase and was found not to interfere with the normal calcification process. Thus phosphatase activity appears essential for normal mineral deposition.

There is, however, no direct evidence, as yet, that PC is a causative factor in mineralisation. Over eight years ago, before the existence of PC in nature was known, Reddi and Huggins (1972) published a report describing the induction of bone cells and bone after bone matrix or bone ash implantation. Alkaline phosphatase and citrate levels were monitored and re-appraisal of this work, in the light of the data presented in this thesis, provides indirect evidence that it may be influential in controlling calcification.

Reddi and Huggins(1972) reported that the subcutaneous transplantation of bone ash resulted in the production of multinucleated cells and, eleven days after implantation, a small rise in citrate levels which eventually fell back to its original level after another fourteen days. Alkaline phosphatase levels remained unchanged and no bone formation was observed.

In contrast, when demineralised bone matrix was implanted then a very large increase in citrate levels was noticeable at day 11, accompanying the histological appearance of bone. Significantly, a considerable elevation of alkaline phosphatase preceded the increase in citrate concentration.

The timing of events after the implantation of demineralised bone matrix - induction of alkaline phosphatase, followed by citrate production and mineralisation - together with the fact that PC is now known to occur naturally, suggests another explanation for these observations. The large rise in citrate concentrations might be due to a prior increase in PC levels at the mineralising site. The phosphatase activity induced prior to calcification ensures that PC is rapidly cleaved removing this inhibitor of HAP formation and supplying one of its precursors - P_i . This proposed appearance of PC at the calcifying site may even correlate with the disappearance of calcium phosphate deposits from the mitochondrial matrix, where it acts to stabilise ACP granules. This model is consistent with the idea that mitochondria is the primary source of PC.

The inability of bone ash implantation to induce calcification can also be explained by this new model. The lack of induction of alkaline phosphatase results in PC remaining essentially unhydrolysed so that inhibition of HAP formation results and P_i levels fail to rise sufficiently to cause mineralisation. The small increase in citrate levels may be due to a limited amount of PC hydrolysis or may even be due solely to PC itself, gives a partial reaction

in the colorimetric (PBA) assay used by Reddi and Huggins (1972) to determine citrate.

Whatever the exact explanation for the failure of bone ash implantation to induce mineralisation, the possibility arises that, after implanting demineralised bone matrix, a sudden increase in PC levels at the calcifying site is a causative factor in mineralisation. This indicates that the Robison hypothesis of bone formation deserves reconsideration, especially in view of the unique properties of PC: its specific location, enzymatic lability and physico-chemical properties.

Clearly investigation of bone is warranted to examine whether or not PC has a special role in this tissue. However, as noted before, PC appears to have a general role in all mitochondria to stabilise calcium phosphate deposits. In this regard another important question arises. Is soft tissue calcification the result of a lack of PC in mitochondria? Such a lack could enable HAP needles to form in mitochondria; once formed this nidus could cause precipitation of Ca^{++} and P_i from physiological fluids due to the insolubility of HAP relative to ACP.

The results presented in this thesis have established PC as a new metabolite and provide stimulus for further research in this interesting area of Ca^{++} metabolism. Although many important aspects of the role of inhibitors of biological calcification, especially concerning PC, have been investigated and valuable results obtained, other interesting and important questions have arisen from the studies reported. Basically, two broad areas are indicated for further study - biochemical

and therapeutic. The results presented suggest PC is located in the mitochondria and preliminary data indicate it may be synthesised in this organelle perhaps under the impetus of Ca^{++} -loading. This area of research needs further investigation as does bone and urine, to confirm whether PC is in fact found in these sources.

The physico-chemical properties of PC indicate PC has therapeutic values where it is stable - blood, for example. However, studies into its in vivo lability indicate analogues of PC, stable to enzymatic degradation, are another new field of research. Information so gained may prove of great importance in the treatment and control of lithiasis as well as many metabolic bone diseases.

REFERENCES

ALI, S.Y., WISBY, A., EVANS, L. and CRAIG-GRAY, J. (1977).

The sequence of calcium and phosphorus accumulation by matrix vesicles.

Cal. Tiss. Res. 27 (Supp.), 490-493.

ALOIA, J.F., ZARIZI, I. and VASWANI, A. (1977).

Combination therapy for osteoporosis.

Metabolism 26, 787-792.

ALTHOFF, J., QUINT, P. and HOHLING, H.J. (1978).

Activation and specificity of alkaline phosphatases of a mineralising collagen-rich system.

Exp. 34, 692-693.

ALTMAN, P.L. and DITTMAR, D.S. (1974).

"Biology Data Book" Volume III. 2nd Edition, p. 1847, Fed. Am. Soc. Exp. Biol., New York.

ANDERSON, H.C. and HSU, H.H.T. (1978).

A new method to measure ⁴⁵Calcium accumulation by matrix vesicles in slices of rachitic growth plate cartilage.

Metab. Bone Dis. Rel. Res. 1, 193-197.

ANNER, B. and MOOSMAYER, M. (1975).

Rapid determination of inorganic phosphate in biological systems by a highly sensitive photometric method.

Anal. Biochem. 65, 305-309.

APPLETON, J. and MORRIS, D.C. (1979).

The use of the potassium pyroantimonate-osmium method as a means of identifying and localising calcium at the ultrastructural level in the cells of calcifying

systems.

J. Histochem. Cytochem. 27, 676-680.

ARLINGER, L. and LUNDIN, H. (1975).

UV-detection of both absorbing and non-absorbing ions in analytical isotachopheresis.

Protides & Biol. Fluids 22, 667-671.

ARNAUD, C.D. (1978).

Calcium homeostasis: regulatory elements and their integration.

Fed. Proc. 37, 2557-2560.

ARSENIS, C. and HUANG, S.M. (1977).

Distribution and immunologic cross reactivity of calcifying cartilage and metaphyseal bone.

Differentiation 8, 183-189.

ASCH, L. (1978).

Calciurie et phosphaturie au cours du jeûne. Action de la calcitonine seule ou associée à une hormonothérapie thyroïdienne.

Rev. de Rhumat. 45, 529-533.

BARKER, L.M. McPHILLIPS, J.J., LAWRENCE, G.D., DOTY, S.B., PALLANTE, S.L., BILLS, C.E., SCOTT, W.W. and HOWARD, J.E. (1970).

Studies of mechanisms of calcification.

I. Properties of urinary derivatives which inhibit cartilage calcification.

II. Electron microscopic observations of the effect of inhibitors on crystal formation.

John Hopkins Med. J. 127, 1-22.

BECKER, G.L., CHUNG-HO, C., GREENAWALT, J.W. and LEHNINGER, A.L. (1974).

Calcium phosphate granules in the hepatopancreas of the blue crab Callinectes sapidus.

J. Cell Biol. 61, 316-326.

BERNARD, G.W. (1978).

Ultrastructural localisation of alkaline phosphatase in initial intramembraneous osteogenesis.

Clin. Orthop. 135, 218-225.

BERTHET-COLOMINAS, C., MILLER, A. and WHITE, S.W. (1979).

Structural study of the calcifying collagen in turkey leg tendons.

Mol. Biol. 134, 431-445.

BEST, P.M. and FORD, L.E. (1978).

Calcium release and membrane voltage changes within muscle cells.

Ann. N.Y. Acad. Sci. 307, 640-641.

BETTS, F., BLUMENTHAL, N.C., POSNER, A.S., BECKER, G.L. and LEHNINGER, A.L. (1975).

Atomic structure of intracellular amorphous calcium phosphate deposits.

Proc. Nat. Acad. Sci. 72, 2088-2090.

BIO-RAD LABORATORIES (1976).

"Chromatography, electrophoresis and membrane filtration".

Bio-Rad Laboratories: p. 14.

BISAZ, J.

—— SCHENCK, R., KUNIN, A.S., RUSSELL, R.G.G., MUHLBAUER, R. and FLEISCH, H. (1975).

Comparative effects of vitamin D deficiency and EHDP administration on the histology and glycolysis of chick epiphyseal and articular cartilage.

Cal. Tiss. Res. 19, 139-148.

- FELIX, R., NEUMAN, W.F. and FLEISCH, H. (1978).
Quantitative determination of inhibitors of calcium phosphate precipitation in whole urine.

Min. Elect. Metab. 1, 74-83.

- BLONDIN, G.A., KESSLER, R.G. and GREEN, D.E. (1977).
Isolation of an electrogenic K^+/Ca^{2+} ionophore from an ionophoroprotein of beef heart mitochondria.

Proc. Natl. Acad. Sci. 74, 3667-3671.

BLUMENTHAL, N.C.

- BETTS, F. and POSNER, A.S. (1975).
Nucleotide stabilisation of amorphous calcium phosphate.
Mat. Res. Bull. 10, 1055-1060.

- BETTS, F. and POSNER, A.S. (1977).
Stabilisation of amorphous calcium phosphate by Mg and ATP.

Cal. Tiss. Res. 23, 245-250.

- POSNER, A.S., SILVERMAN, L.D. and ROSENBERG, L.C. (1979).

Effect of proteoglycans on in vitro hydroxyapatite formation.

Cal. Tiss. Res. 27, 75-82.

BOCEK, P.,

- LEKOVA, K., DEML, M. and JANAK, J. (1976).
Separation of some typical Krebs cycle acids by high-speed isotachopheresis.

J. Chr. 117, 97-109.

- MIEDZIAK, I., DEML, M. and JANAK, J. (1977).
Use of complex formation equilibria in the analytical isotachophoresis of strong electrolyte ions.
Separation of halides and sulphates.
J. Chr. 137, 83-91.
- PAVELKA, S., GRIGELOVA, K., DEML, M. and JANAK, J. (1978a).
Determination of lactic and acetic acids in silage extracts by analytical isotachophoresis.
J. Chr. 154, 356-359.
- DEML, M., KAPLANOVA, B. and JANAK, J. (1978b).
Analytical isotachophoresis. The concept of the separation cavity.
J. Chr. 160, 1-9.
- BODANSZKY, M. and du VIGNEUD, V. (1959).
A method of synthesis of long peptide chains using a synthesis of oxytocin as an example.
J.A.C.S. 81, 5688-5691.
- BONE, H.G., ZERWEKH, T.E., BRITTON, F. and PAK, C.Y.C. (1979).
Treatment of calcium urolithiasis with diphosphonate: efficacy and hazards.
J. Urol. 121, 568-571.
- BONJOUR, J.-P., TROEHLER, U., PRESTON, C. and FLEISCH, H. (1978).
Parathyroid hormone and renal handling of P_i : effect of dietary P_i and diphosphonates.
Am. J. Physiol. 234, F467-F505.

BORIS, A., HURLEY, J.F., TRMAL, T., MALLON, J.P. and MATUSZENSKI, D.S. (1979).

Inhibition of diphosphonate-blocked bone mineralisation.
Evidence that calcitonin promotes mineralisation.
Acta. End. 91, 351-361.

BOSKEY, A.L. (1978).

The role of calcium-phospholipid-phosphate complexes
in tissue mineralisation.
Metab. Bone Dis. Rel. Res. 1, 137-142.

BOSKEY, A.L. (1979).

Models of matrix vesicle calcification.
Inorg. Persp. Biol. Med. 2, 51-92.

BOSKEY, A.L. and POSNER, A.S. (1973).

Conversion of amorphous calcium phosphate to micro-
crystalline hydroxyapatite. A pH dependent solution
- mediated solid-solid conversion.
J. Phys. Chem. 77, 2312-2314.

BOSKEY, A.L. and POSNER, A.S. (1974).

Magnesium stabilization of amorphous calcium phosphate:
a kinetic study.
Mat. Res. Bull. 9, 907-916.

BOSKEY, A.L. and POSNER, A.S. (1977).

The role of synthetic and bone extracted Ca-phospholipid-
 PO_4 complexes in hydroxyapatite formation.
Cal. Tiss. Res. 23, 251-258.

BOYAN-SALYERS, B.D., VOGEL, J.J., RIGGAN, L.J., SUMMER,
F. and HOWELL, R.E. (1978).

Application of a microbial model to biologic calcification.
Metab. Bone Dis. Rel. Res. 1, 143-147.

BOYCE, W.H. and KING, J.S. (1959).

Total non-dialysable solid (TNDS) in human urine.

IV. Electrophoretic properties of RS-1 subfraction.

J. Clin. Invest. 38, 1525-1537.

BOYCE, W.H. and KING, J.S. (1963).

Present concepts concerning the origin of matrix and stones.

Ann. N.Y. Acad. Sci. 104, 563-578.

BOYCE, W.H.,

——— GARVEY, F.K. and NORFLEET, C.M. (1954).

Ion-binding properties of electrophoretically homogeneous mucoproteins of urine in normal subjects and in patients with renal calculus disease.

J. Urol. 72, 1019-1039.

——— KING, J.S. and FIELDEN, M.L. (1962).

Total non-dialyzable solids (TNDS) in human urine.

XII. Immunological detection of a component peculiar to renal calculous matrix and to urine of calculous patients.

J. Clin. Invest. 41, 1180-1189.

BRIGHTON, C.T. and HUNT, R.M. (1978a).

Electron microscopic pyroantimonate studies of matrix vesicles and mitochondria in the rachitic growth plate.

Metab. Bone Dis. Rel. Res. 1, 199-204.

BRIGHTON, C.T. and HUNT, R.M. (1978b).

The role of mitochondria in growth plate calcification as demonstrated in a rachitic model.

J. Bone Joint Surg. 60-A, 630-639.

BRINLEY, F.J. Jnr. (1978).

Comment on the relation between calcium entry and change in ionized calcium.

Ann. N.Y. Acad. Sci. 307, 424-426.

BROULIK, P.D., STEPAN, T. and PACOVSKY, V. (1978).

Increased sensitivity of bone to parathyroid hormone in castrated mice.

Endocrin. Exp. 12, 187-192.

BROWN, J.F. and HINCKLEY, J.O.N. (1975).

Electrophoretic thermal theory. II. Steady-state radial temperature gradients in circular section columns.

J. Chr. 109, 218-224.

BRUNETTE, M.G., VIGNEAULT, N. and DANAN, G. (1978).

A new fluorimetric method for determination of picomoles of inorganic phosphate.

Anal. Biochem. 86, 229-236.

BUSTAMANTE, E., SOPER, J.W. and PEDERSEN, P.L. (1977).

A high-yield preparative method for isolation of rat liver mitochondria.

Anal. Biochem. 80, 401-408.

BYGRAVE, F.L. (1978).

Mitochondria and the control of intracellular calcium.

Biol. Rev. 53, 43-79.

CANNIGGIA, A. and GENNARI, C. (1977).

Effect of ethane hydroxy diphosphonate (etidnonate) on plasma cholesterol and total lipids in man.

Artery 3, 188-192.

CASE, R.M. (1980).

Cell calcium - a new international, interdisciplinary journal.

Cell Calcium 1, 1-6.

CATALINA, F. and CIFUENTES, L. (1970).

Calcium oxalate: Crystallographic analysis in solid aggregates in urinary sediments.

Science 169, 183-184.

CHANDLER, D.E. (1978).

Control of pancreatic enzymatic secretion: a critique on the role of calcium.

Life Sci. 23, 323-333.

CHICKERUR, N.S., TUNG, M.S. and BROWN, W.E. (1980).

A mechanism for incorporation of carbonate into apatite.

Cal. Tiss. Res. 32, 55-62.

CHRISTAKOS, S. and NORMAN, A.W. (1978).

Vitamin D₃-induced calcium binding protein in bone tissue.

Science 202, 70-71.

CLARK, R.B. and DUNCAN, G. (1978).

Two components of extracellularly-recorded photoreceptor potentials in the cephalopod retina: differential effects of Na⁺, K⁺ and Ca⁺⁺.

Biophys. Struct. Mech. 4, 263-271.

COE, F.L. (1978).

Hyperuricosuric calcium oxalate nephrolithiasis.

Kid. Int. 13, 418-426.

COE, F.L., LAWTON, R.L., GOLDSTEIN, R.B. and TEMBE, V. (1975).

Sodium urate accelerates precipitation of calcium oxalate in vitro.

Proc. Soc. Exp. Biol. Res. 149, 926-929.

COSTELLO, L.C. and O'NEILL, J.J. (1969).

A simplified and sensitive method for citrate determination in biological samples.

J. Appl. Physiol. 27, 120-122.

CRAM, S.P. (1978).

Gas chromatography.

Anal. Chem. 50, 213R-243R.

CRASSWELLER, P.O., OREPOULOS, D.G., TOGURI, A., HUSDAN, H., WILSON, D.R. and RAPOPORT, A. (1978).

Studies in inhibitors of calcification and levels of urine saturation with calcium salts in recurrent stone patients.

J. Urol. 120, 6-10.

CRUESS, R.L. and HONG, K.C. (1978).

The long term effect of estrogen administration on the metabolism of male rat bone.

Proc. Soc. Exp. Biol. Med. 159, 368-373.

DALGLEISH, C.E., HORNING, E.C., HORNING, M.G., KNOX, K.L. and YARGER, K. (1966).

A gas-liquid-chromatographic procedure for separating a wide range of metabolites occurring in urine or tissue extracts.

Biochem. J. 101, 792-810.

DANIELS, E. (1978).

The isolation of newly formed bone associated cells in vitro: preliminary observations on origin and morphology.

Exp. Hemat. 6, 48-57.

DAWSON, R.M.C., ELLIOT, D.C., ELLIOT, W.H. and JONES, K.M. (1969).

"Data for biochemical research".

pp. 562-564, Oxford Univ. Press, U.K.

DEGENS, E.T. (1976).

Molecular mechanisms on carbonate, phosphate and silica deposition in the living cell.

Top. Curr. Chem. 64, 1-112.

DELATTE, L.C., MINON, J.L.R., SANTOS, M. and TRABA, M.L.
(1976).

Ectopic renal ossification as nucleus of urinary stones.

J. Urol. 116, 398-401.

DELMOTTE, P. (1979).

Capillary isotachophoresis.

J. Chr. 165, 87-101.

DELUCA, H.F. (1979).

"Vitamin D, Metabolism and Function".

Springer-Verlag, New York.

DENTON, R.M., McCORMACK, J.G. and EDGELL, N.J. (1980).

Role of calcium ions in the regulation of intramitochondrial metabolism. Effects of Na^+ , Mg^{2+} and ruthenium red on the Ca^{++} -stimulated oxidation of oxoglutarate and on pyruvate dehydrogenase activity in intact rat heart mitochondria.

Biochem. J. 190, 107-117.

DERESZEWSKI, G. and HOWELL, D.S. (1978).

The role of matrix vesicles in calcification.

T.I.B.S. 3, 151-153.

DESPOTOVIC, R., FILIPOVIC-VINCEKOVIC, N. and FUREDIMILHOFFER, H. (1975).

Precipitation of calcium phosphates from electrolyte solutions. III. Radiometric studies of the kinetics of precipitation and aging of calcium phosphates.

Cal. Tiss. Res. 18, 13-26.

DICKENS, F. (1941).

III. The citric acid content of animal tissues, with reference to its occurrence in bone and tumour.

Biochem. J. 35, 1011-1023.

DONALDSON, H.H. (1920).

Quantitative studies on the growth of the skeleton of the albino rat.

Am. J. Anat. 26, 237-314.

DOUGHERTY, W.J. (1978).

The occurrence of amorphous mineral deposits in association with the plasma membrane of active osteoblasts in rat and mouse alveolar bone.

Metab. bone Dis. Rel. Res. 1, 119-123.

DRIESSENS, F.C.M. (1980).

Probable phase composition of the mineral in bone.

Z. Naturforsch 35c, 357-362.

DRIESSENS, F.C.M. and VERBEECK, R.M.H. (1980).

Evidence for intermediate metastable states during equilibrium of bone and dental tissue.

Z. Naturforsch 35c, 262-267.

DRIESSENS, F.C.M., van DIJK, J.W.E. and BORGGREVEN, J.M.P.N. (1978).

Biological calcium phosphates and their role in the physiology of bone and dental tissues. I. Comparison and solubility of calcium phosphates.

Cal. Tiss. Res. 26, 126-137.

DYER, T.R. (1965).

"Application of absorption spectroscopy of organic compounds".

Chapter 4, Prentice-Hall Int., London.

DZIAK, R. (1978).

Effects of vitamin D3 metabolites on bone cell calcium transport.

Cal. Tiss. Res. 26, 65-70.

EANES, E.D., TERMINE, J.D. and NYLEN, M.U. (1976).

An electron microscope study of the formation of amorphous calcium phosphate and its transformation to crystalline apatite.

Cal. Tiss. Res. 12, 143-160.

EILON, G. and RAISZ, L.G. (1978).

Comparison of the effects of stimulators and inhibitors of resorption on the release of lysosomal enzymes and radioactive calcium from fetal bone in organ culture.

Endocrin. 103, 1969-1975.

EKSBERG, S., LAGERSTROM, P-O, MODIN, R. and SCHILL, G. (1973).

Ion-pair chromatography of organic compounds.
J. Chr. 83, 99-110.

ELBERS, R., HELDT, H.W., SCHMUCKER, P., SOBOLL, S. and WIESCH, H. (1974).

Measurement of the ATP/ADP ratio in mitochondria and in the extramitochondrial compartment by fractionation of freeze-stopped liver tissue in non-aqueous media.

Hoppe-Seyler's Z. Physiol. Chem. 355, 378-393.

ENDE, J.J. (1978).

Some effects of EHDP and Cl_2 MDP on the activity of alkaline and acid phosphatase in rat bone.

Proc. Kon. Neth. Acad. 81, 150-162.

ENNEVER, J.,

——— BOYAN-SALYERS, B. and RIGGAN, L.J. (1977).

Proteolipids and bone matrix calcification in vitro.

J. Dent. Res. 56, 967-970.

——— VOGEL, J.J. and RIGGAN, L.J. (1978).

Phospholipids of a bone matrix calcification nucleator.

J. Dent. Res. 57, 731-734.

ETTINGER, R.H., GOLDBAUM, L.R. and SMITH, L.H. (1952).

A simplified photometric method for determination of citric acid in biological fluids.

J. Biol. Chem. 199, 531-536.

EVERAERTS, E.M.,

——— PROSE, P. and VERHEGGEN, Th.P.E.M. (1975).

Conductimetric detection during isotachophoresis.

Protides & Biol. Fluids 22, 721-726.

——— GEURTS, M., MIKKERS, F.E.P. and VERHEGGEN, Th.P.E.M. (1976).

Analytical Isotachophoresis.

J. Chr. 119, 129-155.

FAFUNSO, M. and BYERS, M. (1977).

Effect of pre-press treatment of vegetation on the quality of the extracted leaf protein.

J. Sci. Food Agric. 28, 375-380.

FALLON, M., WHYTE, M. and TEITELBAUM, S. (1979).

Stereospecific inhibition of alkaline phosphatase by levamisole prevents in vitro calcification of rachitic rat cartilage.

Cal. Tiss. Int. 28, 147.

FARRINGTON, K., VARGHESE, Z. and MOORHEAD, J.F. (1978).

Vitamin-D analogues and renal function.

Lancet 2, 941.

FAST, D.K.,

—— FELIX, R., NEUMAN, W.F., SALLIS, J. and FLEISCH, H.
(1977).

The effect of diphosphonates on cells in culture.
Cal. Tiss. Res. 27(Supp.), 449-451.

—— FELIX, R., DOWSE, C., NEUMAN, W.F., FLEISCH, H.
(1978).

The effects of diphosphonates on the growth and glycolysis of connective tissue cells in culture.
Biochem. J. 172, 97-107.

FELIX, R. and FLEISCH, H. (1976).

Pyrophosphatase and ATPase of isolated cartilage matrix vesicles.
Cal. Tiss. Res. 22, 1-7.

FELIX, R., HERRMANN, W. and FLEISCH, H. (1978).

Stimulation of precipitation of calcium phosphate of matrix vesicles.
Biochem. J. 170, 681-691.

FINLAYSON, B. (1978).

Physicochemical aspects of urolithiasis.
Kid. Int. 13, 344-360.

FIRSCHEIN, M.E. and URIST, M.R. (1971).

The induction of alkaline phosphatase by extra-skeletal implants of bone matrix.
Cal. Tiss. Res. 7, 108-113.

FISKE, C.H. and SUBBAROW, J. (1925).

Colorimetric determination of phosphorus.
J. Biol. Chem. 66, 375-400.

FISKE, C.H. and SUBBAROW, J. (1929).

Phosphocreatine.

J. Biol. Chem. 81, 629-679.

FISKUM, G. and LEHNINGER, A.L. (1980).

The mechanisms and regulation of mitochondrial Ca^{++} transport.

Fed. Proc. 39, 2432-2439.

FLEISCH, H. (1978).

Inhibitors and promoters of stone formation.

Kid. Int. 13, 361-371.

FLEISCH, H. and BISAZ, S. (1962).

Mechanism of calcification: inhibitory role of pyrophosphate.

Nature 195, 911.

FLEISCH, H. and BISAZ, S. (1964).

The inhibitory effect of pyrophosphate on calcium oxalate precipitation and its relation to urolithiasis.

Exp. 20, 276-277.

FLEISCH, H. and ROBERTSON, W.G. (1970).

The effect of imidodiphosphonate (P-N-P) on the precipitation and dissolution of calcium phosphate in vitro.

Biochem. Biophys. acta 222, 677-680.

FLEISCH, H. and RUSSELL, R.G.G. (1970).

Pyrophosphate and polyphosphate.

In: "The International Encyclopedia of Pharmacology and Therapeutics" (Peters, G. and Radouco-Thomas, C., eds.).

London Pergamon Press, London.

FLEISCH, H. and RUSSELL, R.G.G. (1972).

A review of the physiological and pharmacological effects of pyrophosphate and diphosphonates on bones

and teeth.

J. Dent. Res. 51, 324-332.

FLEISCH, H.,

—— SCHIBLER, D., MAERKI, J. and FROSSARD, I. (1965).

Inhibition of aortic calcification by means of pyrophosphate and polyphosphates.

Nature 207, 1300-1301.

—— RUSSELL, R.G.G., BISAZ, S., TERMINE, J.D. and POSNER, A.S. (1968).

Influence of pyrophosphate on the transformation of amorphous to crystalline calcium phosphate.

Cal. Tiss. Res. 2, 49-59.

—— RUSSELL, R.G.G., BISAZ, S. and MUHLBAUER, R.C. (1970).

The inhibitory effect of phosphonates on the formation of calcium phosphate crystals in vitro and on aortic and kidney calcification in vivo.

Europ. J. Clin. Invest. 1, 12-18.

FLETCHER, E. and MALPRESS, F.H. (1953).

Paper chromatography of phosphate esters.

Nature 171, 838-839.

FORTUNA, R., ANDERSON, H.C., CARTY, R.P. and SAJDERA, S.W. (1978).

The purification and molecular characterisation of alkaline phosphatases from chondrocytes and matrix vesicles of bovine fetal epiphyseal cartilage.

Metab. Bone Dis. Rel. Res. 1, 161-168.

FRANCIS, M.D. (1969).

The inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates.

Cal. Tiss. Res. 3, 151-162.

FRANCIS, M.D.,

——— RUSSELL, R.G.G. and FLEISCH, H. (1969).

Diphosphonates inhibits formation of calcium phosphate crystals in vitro and pathological calcification in vivo.

Science 165, 1265-1266.

——— SLOUGH, C.L., TOFE, A.J. and SILBERSTEIN, E.B. (1976).

Factors affecting uptake and retention of technetium-99m diphosphonate and 99m pertechnate in osseous connective and soft tissue.

Cal. Tiss. Res. 20, 303-311.

FREEDMAN, R.A.,

——— WEISER, M.W. and ISSELBACHER, K.J. (1977).

Calcium translocation by Golgi and lateral-basal membrane vesicles from rat intestine: Decrease in vitamin D-deficient rats.

Proc. Nat. Acad. Sci. 74, 3612-3616.

——— MACLAUGHLIN, J.A. and WEISER, M.M. (1981).

Properties of Ca^{++} uptake and release by Golgi membrane vesicles from rat intestine.

Arch. Biochem. Biophys. 206, 233-241.

FUKUDA, J. and KAMEYAMA, M. (1979).

Enhancement of calcium spikes in nerve cells of adult mammals during neurite growth on tissue culture.

Nature 279, 546-548.

GILL, W.B., SILVERT, M.A. and ROMA, M.J. (1974).

Supersaturation levels and crystallisation rates of calcium oxalate formers from urine of normal humans and stone formers determined by a ^{14}C -oxalate technique.

Invest. Urol. 12, 203-209.

GONZALEZ-DIAZ, P.F., GARCIA-RAMOS, J.V. and SANTO, S.M. (1979).

Composition of apatites in human urinary calculi.
Cal. Tiss. Int. 28, 215-225.

GORDON, L.M., SAUERHEBER, R.D. and ESGATE, J.A. (1978).

Spin label studies on rat liver and heart plasma membranes: effects of temperature, calcium and lanthanum on membrane fluidity.

J. Supramolec. Struct. 9, 299-326.

GORNALL, A.G., BARDAWILL, C.J. and DAVID, M.M. (1949).

Determination of serum proteins by means of the Biuret reaction.

J. Biol. Chem. 177, 751-766.

GREENAWALT, J.W., ROSSI, C.S. and LEHNINGER, A.L. (1964).

Effect of active accumulation of calcium and phosphate ions on the structure of rat liver mitochondria.

J. Cell. Biol. 23, 21-38.

GREGER, R., LANG, F., OBELEITHNER, H. and DEETJEN, P. (1978).

Outflux of ⁴⁵-calcium along the rat nephron.

Adv. Exp. Med. Biol. 103, 125-128.

GRUSCHKA, E., DURST, H.D. and KIKTA, E.J. (1975).

Liquid chromatographic separation and detection of nanogram quantities of biologically important dicarboxylic acids.

J. Chr. 112, 673-678.

GULYI, M.F.,

——— PECHENOVA, T.N. and DVORNIKOVA, P.D. (1963).

Formation of acetyl phosphate in liver homogenates.

Dok. Akad. Nauk. S.S.S.R. 146, 933-935.

- PECHENOVA, T.N. and MATUSEVICH, L.T. (1965).
Pathways and enzymes involved in conversion of citric acid into acetylphosphate in animal tissues.
Ukr. Biokhim. Zhur. 37, 56-61.
- HABENER, J.F. and KRONENBERG, H.M. (1978).
Parathyroid hormone biosynthesis: structure and function of biosynthetic precursors.
Fed. Proc. 37, 2561-2566.
- HAGERMAN, A.E. and BUTLER, L.G. (1978).
Protein precipitation method for the quantitative determination of tannins.
J. Agric. Food Chem. 26, 809-912.
- HALLSON, R.G. and ROSE, G.A. (1979).
Uromicoids and urinary stone formation.
Lancet 1, 1000-1002.
- HAMILTON, J.W. and HOLDSWORTH, E.S. (1970).
The release of ^{45}Ca mitochondria of chicken intestinal mucosa by calcium binding protein.
Biochem. Biophys. Res. Comm. 40, 1325-1330.
- HARADA, E., LAYCHOCK, S.G. and RUBIN, R.P. (1978).
Parathyroid hormone induced stimulation of calcium uptake by renal microsomes.
Biochem. Biophys. Res. Comm. 84, 396-402.
- HARRIS, E.J. (1978).
The importance of CO_2 for Ca^{++} uptake by some mitochondria.
Nature 274, 820-821.
- HASHIZUME, T. and SASAKI, Y. (1966).
Gas chromatography of sugar phosphate.
Anal. Biochem. 15, 346-350.

HAUSCHKA, P.V. and REID, M.L. (1978a).

Timed appearance of a calcium-binding protein containing γ -carboxyglutamic acid in developing chick bone.

Dev. Biol. 65, 426-434.

HAUSCHKA, P.V. and REID, M.L. (1978b).

Vitamin K dependence of a calcium-binding protein containing γ -carboxyglutamic acid in chicken bone.

J. Biol. Chem. 253, 9063-9068.

HAUSCHKA, P.V.,

—— LIAN, J.B. and GALLOP, P.M. (1975).

Direct identification of the calcium-binding amino acid, γ -carboxyglutamate, in mineralised tissue.

Proc. Nat. Acad. Sci. 12, 3925-3929.

—— LIAN, J.B. and GALLOP, P.M. (1978).

Vitamin K and mineralisation.

T.I.B.S. 3, 75-78 (1978).

HAUSMANN, E., BISAZ, S., RUSSELL, R.G.G. and FLEISCH, H. (1970).

The concentration of inorganic pyrophosphate in human saliva and dental calculus.

Arch. oral. Biol. 15, 1389-1392.

HAUTMANN, R., LEHMANN, A. and KOMOR, S. (1980).

Calcium and oxalate concentrations in human renal tissues: the key to the pathogenesis of stone formation?
J. Urol. 123, 317-319.

HAY, D.I., MORENO, E.C. and SCHLESINGER, D.H. (1979).

Phosphoprotein - inhibitors of calcium phosphate precipitation from salivary secretions.
Inorg. Perspect. Biol. Med. 2, 271-285.

HEARN, M.T.W. and HANCOCK, W.S. (1979).

Ion pair partition reversed phase HPLC.
T.I.B.S. 4, 58-62.

HECHT, S.M. and KOZARICH, J.W. (1973).

A chemical synthesis of adenosine - 5-[γ - ^{32}P]-triphosphate.
Biochem. Biophys. acta 331, 307-309.

HEERSCHE, J.N.M. (1978).

Mechanism of osteoclastic bone resorption: a new hypothesis.
Cal. Tiss. Res. 26, 81-84.

HEERSCHE, J.N.M., HEYBOER, M.P.M. and NG, B. (1978).

Hormone-specific suppression of adenosine 3',5'-monophosphate responses in bone in vitro during prolonged incubation with parathyroid hormone, prostaglandin E, and calcitonin.
Endocrin. 103, 333-340.

HELLMAN, B. (1979).

Calcium and pancreatic beta-cell fraction. 5. Mobilisation of a glucose-stimulated pool of intracellular 45 -calcium by metabolic inhibitors and the ionophore A23187. Acta End. 90, 624-636.

HENRY, J.P. and NINIO, M. (1978).

Control of Ca^{++} -triggered bioluminescence of Veretillum cyanomorum lumisenes. Biochem. Biophys. acta 504, 40-48.

HERBST, D.M. and DEAMER, D.W. (1977).

Calcium-dependent adenosine triphosphate activity preservation in isolated sarcoplasmic reticulum. Physiol. Chem. Phys. 9, 123-129.

HIGGINS, H. and VON BRAND, T. (1966).

Separation of lactic acid and some Krebs cycle acids by TLC. Anal. Biochem. 15, 122-126.

HOHLING, H.J., BARCKHAUS, R.H. and KREFTING, E.R. (1980).

Hard tissue formation in collagen-rich systems: calcium phosphate nucleation and organic matrix. T.I.B.S. 5, 8-11.

HOLICK, M.F. and CLARK, M.B. (1978).

The photobiogenesis and metabolism of vitamin D. Fed. Proc. 37, 2567-2574.

HOWARD, J.E. (1976).

Studies on urinary stone formation: a saga of clinical investigation. Johns Hopkins Med. J. 139, 239-252.

HOWARD, J.E., THOMAS, W.C., BARKER, L.M., SMITH, L.H.
and WADKINS, C.L. (1967).

The recognition and isolation from urine and serum
of a peptide inhibitor to calcification.

Johns Hopkins Med. J. 120, 119-136.

HOWELL, D.S. and CARLSON, L. (1968).

Alterations in the composition of growth cartilage
septa during calcification studied by microscopic
X-ray element analysis.

Exp. Cell Res. 51, 185-195.

HSU, H.H.T., CECIL, R.N.A. and ANDERSON, H.C. (1978).

Role of adenosine triphosphate, phospholipids and
vesicular structure in the calcification of isolated
and reconstructed matrix vesicles.

Metab. Bone Dis. Rel. Res. 1, 169-172.

HUKINS, D.W.L. (1978).

Bone stiffness explained by the liquid crystal model
for the collagen fibril.

J. Theor. Biol. 71, 661-667.

ITO, H. and COE, F.L. (1977).

Acidic peptide and polyribonucleotide crystal growth
inhibitors in human urine.

Am. J. Physiol. 233, F455-F463.

JACOBSON, B.S. and BRANTON, D. (1977).

Plasma membrane: rapid isolation and exposure of
the cytoplasmic surface by use of positively charged
beads.

Science 195, 302-304.

JONES, G.B. (1967).

Estimation of microgram quantities of citrate in

biological fluids.

Anal. Biochem. 21, 286-292.

JOHNSON, L.F. and JANKOWSKI, W.C. (1972).

Carbon-13 NMR spectra.

p 172, Wiley, New York.

de JONG, L., HOLTROP, M. and KROON, A.M. (1978).

Oxidative phosphorylation in mitochondria isolated from small intestinal epithelium of thiamphenicol-treated rats and control rats.

Biochem. Biophys. acta 501, 405-414.

JUNG, A.,

——— RUSSELL, R.G.G., BISAZ, S., MORGAN, D.B. and FLEISCH, H. (1970).

Fate of intravenously injected pyrophosphate - ^{32}P in dogs.

Am. J. Physiol. 218, 1757-1764.

——— BISAZ, S. and FLEISCH, H. (1973).

The binding of pyrophosphate and two diphosphonates by hydroxyapatite crystals.

Cal. Tiss. Res. 11, 269-280.

KAWASAKI, T. (1978).

Theory of chromatography of rigid molecules on hydroxyapatite columns with small loads. IV. Estimation of the adsorption energy of nucleoside polyphosphates.

J. Chr. 151, 95-121.

KAY, H.D. (1928).

CVIII. The phosphatases of mammalian tissues.

Biochem. J. 22, 857-866.

KEELEY, F.W. (1977).

The extraction and partial characterisation of proteins released by decalcification from calcified human aortic

plaques.

Biochem. Biophys. acta 494, 384-394.

KEELEY, F.W. (1978).

Identification of serine phosphate in a protein fraction occluded in the mineralised matrix of calcified human aorta.

Biochem. Biophys. Res. Comm. 85, 934-937.

KENNOKI, F., MIZUHIRA, V., KONJIKI, T. and KAWAI, H. (1978).

Elemental analysis of urinary tract calculi using an electron microscope with electron probe X-ray micro-analyser.

Acta Histochem. Cytochem. 11, 387-398.

KEPNER, B.L. and HERCULES, D.M. (1963).

Fluorimetric determination of calcium in blood serum.

Anal. Chem. 35, 1238-1240.

KHAIRI, M.R.A., ALMAN, R.D. and DEROSA, G.P. (1977).

Sodium etidronate in the treatment of Paget's disease of bone. A study of long-term results.

Ann. Intern. Med. 87, 656-663.

KHWAJA, T.A. and REESE, C.B. (1971).

The phosphorylation of nucleosides with o-phenylene phosphorochloridate and o-phenylene phosphate.

Tetra. 27, 6189-6198.

KHWAJA, T.A., REESE, C.B. and STEWART, J.C.M. (1970).

A convenient general procedure for the conversion of alcohols into their monophosphate esters.

J. C. S. (C), 2092-2106.

KHYM, J.T. (1975).

An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchangers.

Clin. Chem. 21, 1245-1252.

KIEBOOM, A.P.G., VIJVERBERG, C.A.M., PETERS, J.A. and VAN BEKKUM, H. (1978).

Complexation of acetate, glycolate, lactate, malate and citrate anions with lanthanide (III) cations in aqueous solution as studied by NMR spectroscopy.

J. Royal Neth. Chem. Soc. 315-316.

KING, J.S. (1971).

Currents in renal stone research.

Clin. Chem. 17, 971-982.

KING, J.S. and BOYCE, W.H. (1962).

Some experiments on the relations of urinary macromolecules to stone growth.

Rein Foie 4, 1-11.

KING, K. (1978).

Distribution of γ -carboxyglutamic acid in calcified tissues.

Biochem. Biophys. acta 542, 542-546.

KINNE, R., HASSE, W., GMAJ, P. and MURER, H. (1977).

ATP-hydrolysis as driving force for transport processes in isolated renal plasma membrane vesicles.

Curr. Prob. Clin. Biochem. 8, 178-185.

KISTLER, A. (1978).

Inhibition of vitamin A action in rat bone cultures by inhibitors of RNA and protein synthesis.

Exp. 34, 1159-1161.

KISTLER, A. (1979).

Retinoic acid induced proteoglycan release and cartilage resorption in rat bone culture are age dependent and inhibited by EDTA.

Wilhelm Roux's Arch. 187, 59-71.

KOMISSARENKO, S.V., GULAYA, N.M. and GUBENKO, E.P. (1977).

Inhibition of mouse spleen inorganic pyrophosphate by methylene diphosphonic acid.

Biokhim. 42, 238-242.

KORHONEN, L.K., HAMALAINEN, M. and KAIVOSOJA, M. (1978).

Inorganic pyrophosphatase activity distinct from alkaline phosphatase in rat bone.

Clin. Orthop. 128, 332-339.

KOSKINEN, E.V., NIEMINEN, R.A., LINDHOLM, R.V., PURANEN, J. and ATTILA, U. (1977).

Human growth hormone in bone regeneration of non-healing fractures.

Cal. Tiss. Res. 22(Supp.), 521-523.

KRANE, S.M. and GLIMCHER, M.J. (1962).

Transphosphorylation from nucleoside di- and triphosphates by apatite crystals.

J. Biol. Chem. 237, 2991-2998.

KRESAK, M., MORENO, E.C., ZAHRAPNIK, R.T. and HAY, D.I. (1977).

Adsorption of amino acids onto hydroxyapatite.

J. Coll. Int. Sci. 59, 283-292.

LAGERSTROM, P-O. (1975).

Ion pair chromatography of carboxylic acid.

Acta Pharm. Suec. 12, 215-234.

LAMBERT, C.C. and EPEL, D. (1979).

Calcium-mediated mitochondrial movement in ascidian sperm during fertilization.

Dev. Biol. 69, 296-309.

LANDIS, W.J. and GLIMCHER, M.J. (1978).

Electron diffraction and electron probe microanalysis of the mineral phase of bone tissue prepared by anhydrous techniques.

J. Ultrastruct. Res. 63, 188-223.

LANZETTA, R.A., ALVAREZ, L.J., REINACH, P.S. and CANDIA, O.A. (1979).

An improved assay for nanomole amounts of inorganic phosphate.

Anal. Biochem. 100, 95-97.

LAOUARI, D., PAVLOVITCH, H., DECENEUX, G. and BALSAN, S. (1980).

A vitamin D-dependent calcium-binding protein in rat skin.

F.E.B.S. Lett. 111, 285-289.

LECLERCQ-MEYER, V., MARCHAND, J. and MALAISSE, W.J. (1978).

The role of calcium in glucagon release. Studies with verapamil.

Diabetes 27, 996-1004.

LEE, N.H. and SHAPIRO, I.M. (1978).

Ca^{++} transport by chondrocyte mitochondria of the epiphyseal growth plate.

J. Memb. Biol. 41, 349-360.

LEE, S.L., VEIS, A. and GLONEK, T. (1977).

Dentin phosphoprotein: an extracellular calcium binding protein.

Biochem. 16, 2971-2979.

LEES, S. (1979).

A model for the distribution of HAP crystallites in bone - an hypothesis.

Cal. Tiss. Int. 27, 53-56.

LEHNINGER, A.L. (1970).

Mitochondria and calcium ion transport.

Biochem. J. 119, 129-138.

LEHNINGER, A.L. (1977).

Mitochondria and biological mineralisation processes: an exploration.

Horiz. Biochem. Biophys. 4, 1-30.

LEHNINGER, A.L., REYNAFARJE, B., VERCESI, A. and TEW, W.P. (1978).

Transport and accumulation of calcium in mitochondria.

Ann. N.Y. Acad. Sci. 307, 160-176.

LEIBMAN, K.C. and HINDMAN, J.O. (1964).

Modification of the Fujiwara reaction for determination of polyhalogenated organic compounds.

Anal. Chem. 36, 348-351.

LEONARD, F. and SCULLIN, R.L. (1969).

New mechanism for calcification of skeletal tissues.

Nature 224, 1113-1114.

LEWINSON, D. and SILBERMAN, M. (1978).

Chondrocyte involvement in condylar cartilage calcification utilizing potassium pyroantimonate-osmium tetroxide.

Metab. Bone Dis. Rel. Res. 1, 243-250.

LEWIS, A.M., THOMAS, W.C. and TOMIT, A. (1966).

Pyrophosphate and the mineralising potential of urine.

Clin. Sci. 30, 389-397.

LIAN, J.B. and FRIEDMAN, P.A. (1978).

The vitamin D-dependent synthesis of γ -carboxyglutamic acid by bone microsomes.

J. Biol. Chem. 253, 6623-6626.

LIAN, J.B., HAUSCHKA, P.V. and GALLOP, P.M. (1978).

Properties and biosynthesis of a vitamin K-dependent calcium binding protein in bone.

Fed. Proc. 37, 2615-2620.

LINDBERG, O. and ERNSTER, L. (1956).

Detection of organic phospho compounds.

Meth. Biochem. Anal. III, 1-33.

LINDEN, G. and ALAIS, C. (1976).

Phosphatase alcaline due lait de vaches. II. Structure sous-unitaire, nature metalloproteique et parametres cinetiques.

Biochem. Biophys. acta 429, 205-213.

LOHMANDER, S. and HJERPE, A. (1975).

Proteoglycans of mineralising rib and epiphyseal cartilage.

Biochem. Biophys. acta 404, 93-109.

LONSDALE, K. (1968).

Epitaxy as a growth factor in urinary calculi and gallstones.

Nature 217, 56-58.

LOWRY, O.H., PASSONNEAU, J.V., SCHULZ, D.W. and ROCK, M.K. (1961).

The measurement of pyridine nucleotides by enzymatic cycling.

J. Biol. Chem. 236, 2746-2755.

LUBEN, R.A. and WADKINS, C.L. (1971).

Studies of the relationship of proton production and calcification of tendon matrix in vitro.

Biochem. 10, 2183-2189.

LUTHRA, R. and OLSON, M.S. (1976).

Studies of mitochondrial calcium movements using chlorotetracycline.

Biochem. Biophys. acta 440, 744-758.

McGANN, T.C.A. (1979).

Calcium phosphate analogues in milk and bone salts.

Biochem. Soc. Trans. 7, 51-52.

MACINTYRE, I., COLSTON, K.N., SZELKE, M. and SPANOS, E. (1978).

A survey of the hormonal factors that control calcium homeostasis.

Ann. N.Y. Acad. Sci. 307, 345-355.

MACLENNAN, D.H. and KLIP, A. (1979).

Calcium transport and release by sarcoplasmic reticulum: a mini-review.

Soc. Gen. Physiol. Ser. 33, 61-75.

MACPARTLIN, J., SKRABANE, K.P. and POWELL, D. (1978).

Early effects of parathyroid hormone in rat calvarian bone alkaline phosphatase.

Endocrin. 103, 1573-1578.

MAHIN, D.J. and LOFBERG, R.T. (1966).

A simplified method of sample preparation for determination of tritium, carbon-14, or sulfur-35 in blood or tissue by liquid scintillation counting.

Anal. Biochem. 16, 500-509.

MANTSCH, H.H. and SMITH, I.C.P. (1972).

Fourier-transformed ^{13}C NMR spectra of polyuridylic acid, uridine and related nucleotides - the use of ^{31}P OC ^{13}C couplings for conformational analysis.

Biochem. Biophys. Res. Comm. 46, 808-815.

MARCHE, P., THOMASSET, M. and CUISINIER-GLEIZES, P. (1978)

"Calcium-binding protein" intestinale: une expression moléculaire de l'action hormonales de la vitamine D₃.

Ann. d'Endocrin. (Paris) 39, 219-220.

MASSINE, P., KASER-GLANZMANN, R. and LUSCHER, E.F. (1978)

Movement of calcium ions and their roles in the activation of platelets.

Thromb. Haemost. 40, 212-218.

MATTHEWS, J.L., MARTIN, J.H., SAMPSON, H.W., KUNIN, A.S.

and ROAN, J.H. (1970).

Mitochondrial granules in the normal and rachitic rat epiphysis.

Cal. Tiss. Res. 5, 91-99.

MAXIMOV, E.E., ZAREVALOVA, N.P. and MITIN, Y.V. (1978).

On the calcium-binding ability of the synthetic evolutionary ancestor of calcium-binding protein.

F.E.B.S. Lett. 88, 80-82.

MEANS, A.R. and DEDMAN, J.R. (1980).

Calmodulin in endocrine cells and its multiple roles in hormone action.

Molec. Cell. Endocrin. 19, 215-227.

MESSER, H.H., ARMSTRONG, W.D. and SINGER, L. (1973).

Fluoride, parathyroid hormone and calcitonin: interrelationships in bone calcium metabolism.

Cal. Tiss. Res. 13, 217-225.

MEYER, J., BOLEN, R.J. and STAKELUM, J.J. (1959).

The synthesis of citric acid phosphate.

J.A.C.S. 81, 2094-2096.

MEYER, J.L. and ANGINO, E.E. (1977).

The role of trace metals in calcium urolithiasis.

Invest. Urol. 14, 347-350.

MEYER, J.L. and EANES, E.D. (1978a).

A thermodynamic analysis of the amorphous to crystalline calcium phosphate transformation.

Cal. Tiss. Res. 25, 59-68.

MEYER, J.L. and EANES, E.D. (1978b).

A thermodynamic analysis of the secondary transition in the spontaneous precipitation of calcium phosphate.

Cal. Tiss. Res. 25, 209-216.

MEYER, J.L. and NANCOLLAS, G.H. (1973).

The influences of multidentate organic phosphates on the crystal growth of hydroxyapatite.

Cal. Tiss. Res. 13, 295-303.

MEYER, J.L. and SELINGER, A.H. (1980).

Effect of citrate on calcium phosphate phase transitions.

Min. Elect. Metab. 3, 207-216.

MEYER, J.L. and SMITH, L.H. (1975 a).

Growth of calcium oxalate crystals. I. A model for urinary stone growth.

Invest. Urol. 13, 31-35.

MEYER, J.L. and SMITH, L.H. (1975 b).

Growth of calcium oxalate crystals. II. Inhibition by natural urinary crystal growth inhibitors.

Invest. Urol. 13, 36-39.

MEYER, J.L., McCALL, J.T. and SMITH, L.H. (1974).

Inhibition of calcium phosphate crystallization by nucleoside phosphatases.

Cal. Tiss. Res. 15, 287-293.

MEYERS, T.C., NAKAMURA, K. and FLESHER, J.W. (1963).

Phosphoric acid analogs of nucleoside phosphates.

I. The synthesis of 5'-adenylyl methylene diphosphonate, a phosphoric acid analog of ATP.

J.A.C.S. 85, 3292-3295.

MICHAEL, W.R., KING, W.R. and WAKIM, J.M. (1972).

Metabolism of disodium-1-hydroxy-1,1-diphosphonate (Disodium etidronate) in the rat, rabbit, dog and monkey.

Toxic. App. Pharm. 21, 503-515.

MILLER, J.D., RANDOLPH, A.D. and DRACH, G.W. (1977).

Observations upon calcium oxalate crystallization kinetics in simulated urine.

J. Urol. 117, 342-345.

MILLER, S.C. and JEE, W.S. (1977).

The comparative effects of dichloromethylene disphos-

phonate (Cl_2 MDP) and ethane 1-hydroxy 1,1-diphosphonate (EHDP) on growth and modelling of the rat tibia.

Cal. Tiss. Res. 23, 207-214.

MILLER, S.C., JEE, W.S.S., KIMMEL, D.B. and WOODBURY, L. (1977).

Ethane-1-hydroxy-1,1-diphosphonate (EHDP) effects on incorporation and accumulation of osteoclast nuclei.

Cal. Tiss. Res. 22, 243-252.

MOORE, L. and PASTAN, I. (1978).

Energy-dependent calcium uptake by fibroblast microsomes.

Ann. N.Y. Acad. Sci. 307, 177-194.

MORIARTY, C.M. (1978).

Role of calcium in the regulation of adeno-hypophysial hormone release.

Life Sci. 23, 185-194.

MORRISSEY, R.L., ZOLOCK, D.T., BIKLE, D.D., EMPSON, R.N. and BUCCI, T.J. (1978).

Intestinal response to 1α , 25-dihydroxycholecalciferol.

I. RNA polymerase, alkaline phosphatase, calcium and phosphate uptake, in vitro and in vivo calcium transport and accumulation.

Biochem. Biophys. acta 538, 23-33.

MORTON, R.K. (1958).

The phosphotransferase activity of phosphatases.

2. Studies with purified alkaline phosphomono-esterase and some substrate-specific phosphatase.

Biochem. J. 70, 139-155.

NAKAGAWA, Y., KAISER, E.T. and COE, F.L. (1978).

Isolation and characterisation of calcium oxalate crystal growth inhibitors from human urine.

Biochem. Biophys. Res. Comm. 84, 1038-1044.

NANCOLLAS, G.H. and MOHAN, M.S. (1970).

The growth of hydroxyapatite crystals.

Arch. oral Biol. 15, 731-745.

NEUMAN, W.F. and NEUMAN, M.W. (1958).

"The chemical dynamics of bone mineral".

Univ. Chicago Press, Chicago.

NEUMAN, W.F., BROMMAGE, R. and MYERS, C.R. (1977).

The measurement of Ca^{++} effluxes from bone.

Cal. Tiss. Res. 24, 113-117.

NEUMAN, M.W., NEUMAN, W.F. and FLEISCH, H. (1978).

The influx of pyrophosphate ion into calvaria in vitro.

Cal. Tiss. Res. 25, 19-21.

NEWBURY, H.J. and POSSINGHAM, J.V. (1977).

Factors affecting the extraction of intact ribonucleic acid from plant tissues containing phenolic compounds.

Plant Physiol. 60, 543-547.

NICHOLSON, J.D. (1978a).

Derivative formation in the quantitative gas-chromatographic analysis of pharmaceuticals.
Part I.

Analyst 103, 1-28.

NICHOLSON, J.D. (1978b).

Derivative formation in the quantitative gas-chromatographic analysis of pharmaceuticals.

Part II.

Analyst 103, 193-222.

NISHIDA, E. (1978).

Effects of solution variables on the calcium sensitivity of the microtubule assembly system.

J. Biochem. 84, 507-512.

NORMAN, A.W. and ROSS, F.P. (1979).

Vitamin D seco-steroids: unique molecules with both hormone and possible membranophilic properties.

Life Sci. 24, 759-770.

OGAWA, S., ROTTENBERG, H., BROWN, T.R., SHULMAN, R.G., CASTILLO, C.L. and GLYNN, P. (1978).

High-resolution ^{31}P NMR study of rat liver mitochondria.

Proc. Nat. Acad. Sci. 75, 1796-1800

OHATA, M. and PAK, C.Y.C. (1973).

The effect of diphosphonate on calcium phosphate crystallization in urine in vitro.

Kid. Int. 4, 401-406.

OHATA, M. and PAK, C.Y.C. (1974).

Preliminary studies of the treatment of nephrolithiasis (calcium stones) with diphosphonate.

Metabolism 23, 1167-1173.

OHNISHI, S.T. (1978).

A new method of separating inorganic orthophosphate from phosphoric esters and anhydrides by an immobilized catalyst column.

Anal. Biochem. 86, 201-213.

OMDAHL, J.L. and DELUCA, H.F. (1973).

Regulation of vitamin D metabolism and function.
Physiol. Rev. 53, 327-372.

O'NEILL, J.J. and SAKAMOTO, T. (1969).

Fluorimetric micro-determination of citrate in tissue extracts.

Anal. Biochem. 29, 357-380.

ONO, Y., NAGATA, N. and KIMURA, N. (1978).

Effects of adrenergic and cholinergic stimulation on cyclic nucleotide metabolism in bone of young rats.
Endocrin. Japon. 25, 477-484.

ORNOY, A., GOODWIN, D., NOFF, D. and EDELSTEIN, S. (1978).

24,25-dihydroxy vitamin D is a metabolite of vitamin D essential for bone formation.
Nature 276, 517-519.

OSTROWSKI, K., DZIEDZIC-GOCLAWSKA, A. and SLOWOWSKI, A. (1975).

Analysis of the crystallinity of calcium phosphate deposits in rat liver mitochondria by electron spin resonance spectroscopy.
F.E.B.S. Lett. 60, 410-413.

PAK, C.Y.C. (1978).

Calcium urolithiasis: is it analogous to bone formation?
Cal. Tiss. Res. 26, 195-197.

PAK, C.Y.C. and ARNOLD, L.H. (1975).

Heterogeneous nucleation of calcium oxalate by seeds of monosodium urate.
Proc. Soc. Exp. Biol. Med. 149, 930-932.

PAK, C.Y.C. and HOLT, K. (1976).

Nucleation and growth of brushite and calcium oxalate in urine of stone formers.

Metabolism 25, 665-673.

PAK, C.Y.C., HAYASHI, Y. and ARNOLD, L.H. (1976).

Heterogeneous nucleation with urate, calcium phosphate and calcium oxalate.

Proc. Soc. Exp. Biol. Med. 153, 83-87.

PALMER, J.K. and LIST, D.M. (1973).

Determination of organic acids in foods by liquid chromatography.

J. Agr. Food Chem. 21, 903-906.

PALMOSKI, M. and BRANDT, K. (1978).

Effects of diphosphonates on glycosaminoglycan synthesis and proteoglycan aggregation in normal adult articular cartilage.

Arth. Rheum. 21, 942-949.

PANFILI, E., SANDRI, G., SOTTOCASA, G.L., LUNAZZI, G., LIUT, G. and GRAZIOISI, G. (1976).

Specific inhibition of mitochondrial Ca^{++} -transport by antibodies directed to the Ca^{++} -binding glycoprotein.

Nature 264, 185-186.

PARKER, C.M., SHARMA, R.P. and SHUPE, J.L. (1979).

The interaction of dietary vitamin C, protein and calcium with fluoride: effects in guinea pigs in relation to breaking and radiodensity of bone.

Clin. Toxicol. 15, 301-311.

PARRY, H.M.III and AVIOLI, L.V. (1981).

Effect of sodium etidronate and phenytoin on Pagetic

bone.

Cal. Tiss. Res. 33, 285-286.

PASSONEAU, J.V. and BROWN, J.G. (1974).

Fluorimetric determination.

In: "Methods of Enzymatic Analysis" Vol. III pp
1565-1569.

H.U. Bergmeyer (ed.).

Academic Press, N.Y. and London.

PELLEGRINO, E.D. and BILTZ, R.M. (1978).

Calcium phosphate carbonate transformations in renal
calculi.

Cal. Tiss. Res. 26, 191-193.

PENNY, C.L. (1976).

A simple micro-assay for inorganic phosphate.

Anal. Biochem. 75, 201-210.

PERKINS, H.R. and WALKER, P.G. (1958).

The occurrence of pyrophosphate in bone.

J. Bone Joint Surg. 40B, 333-339.

POSNER, A.S. (1969).

Crystal chemistry of bone mineral.

Physiol. Rev. 49, 760-792.

POSNER, A.S. (1973).

Bone mineral of the molecular level.

Fed. Proc. 32, 1933-1937.

POSNER, A.S. and BETTS, F. (1975).

Synthetic amorphous calcium phosphate and its relation
to bone mineral structure.

Acc. Chem. Res. 8, 273-281.

POSNER, A.S., BETTS, F. and BLUMENTHAL, N.C. (1978).

Properties of nucleating systems.

Metab. Bone Dis. Rel. Res. 1, 179-184.

PRICE, P.A., OTSUKA, A.S., POSER, J.W., KRISTAPONIS, J.
and RAMAN, N. (1976).

Characterization of a γ -carboxyglutamic acid-containing
protein from bone.

Proc. Nat. Acad. Sci. 73, 1447-1451.

QUITTNER, C. and WADKINS, C.L. (1978).

A macromolecular inhibitor of in vitro calcification
of tendon matrix.

Cal. Tiss. Res. 25, 161-168.

RAEYMAEKERS, L., AGOSTINI, B. and HASSELBACH, W. (1981).

The formation of intravesicular calcium phosphate
deposits in microsomes of smooth muscle. A comparison
with sarcoplasmic reticulum of skeletal muscle.

Histochem. 70, 139-150.

RAO, M.V.R., AGARWAL, J.S. and TANEJA, O.P. (1978).

Electron optical investigation on the morphology of
the colloidal matrix of urinary calculi.

Invest. Urol. 15, 452-455.

RASMUSSEN, H. (1977).

Calcium and cyclic nucleotides as universal second
messengers.

Soc. Gen. Physiol. Ser. 32, 243-268.

RASMUSSEN, H. and GOODMAN, D.B.P. (1977).

Relationships between calcium and cyclic nucleotides
in cell activation.

Physiol. Rev. 57, 421-509.

REDDI, A.H. and HUGGINS, C.B. (1972).

Citrate and alkaline phosphatase during transformation of fibroblasts by the matrix.

Proc. Soc. Exp. Biol. Med. 140, 807-810.

REDDI, A.H.,

—— HASCALL, V.C. and HASCALL, G.K. (1978).

Changes in proteoglycan types during matrix-induced cartilage and bone development.

J. Biol. Chem. 253, 2429-2436.

—— MEYER, J.L., TEW, W.P., HOWARD, J.E. and LEHNINGER, A.L. (1980).

Influences of phosphocitrate, a potent inhibitor of hydroxyapatite crystal growth, on mineralization of cartilage and bone.

Biochem. Biophys. Res. Comm. 97, 154-159.

RESNICK, M.I. and BOYCE, W.H. (1978).

Spherical calcium bodies in stone-forming urine.

Invest. Urol. 15, 449-451.

REVIS, N. and MARUSIC, N. (1979).

Sequestration of $^{45}\text{Ca}^{++}$ by mitochondria from rabbit heart, liver, and kidney after doxorubicin or digoxin/doxorubicin treatment.

Exp. Mel. Path. 31, 440-451.

REY, C., TROMBE, J.C. and MONTEL, G. (1977).

Sur un premier modele des relations, a l'echelle moleculaire de la glycine, entre constituants organiques et mineraux des tissus mineralises.

Bull. Group int. Rech. Sc. Stomat. et Odont 20, 5-16.

RIBOVICH, M.L. and DELUCA, H.F. (1978).

The effect of dietary calcium and phosphorus on intestinal calcium absorption and vitamin D metabolism.

Arch. Biochem. Biophys. 188, 145-157.

ROBERTSON, W.G. and FLEISCH, H. (1970).

The effect of imidodiphosphate (P-N-P) on the precipitation and dissolution of calcium phosphate in vitro.

Biochem. Biophys. acta 222, 677-680.

ROBERTSON, W.G. and PEACOCK, M. (1972).

Calcium oxalate crystallization and inhibitors of crystallization in recurrent renal stone-formers.

Clin. Sci. 43, 499-506.

ROBERTSON, W.G.,

——— PEACOCK, M. and NORDIN, B.E.C. (1971).

Calcium oxalate crystalluria and urine saturation in recurrent renal stone-formers.

Clin. Sci. 40, 365-374.

——— PEACOCK, M. and KNOWLES, C.F. (1973).

Calcium oxalate crystalluria and inhibition of crystalluria in recurrent renal stone formers.

In: "Urinary Calculi", Int. Symp. Renal Stone Res., Madrid, 1972.

pp 302-306, Karger, Basel.

——— PEACOCK, M., HEYBURN, P.J., MARSHALL, D.H. and CLARK, P.B. (1978).

Risk factors in calcium stone disease of the urinary tract.

Brit. J. Urol. 50, 449-454.

——— PEACOCK, M. and HODGKINSON, A. (1979).

Dietary changes and the incidence of urinary calculi in the U.K. between 1958 and 1976.

J. Chron. Dis. 32, 469-476.

ROBISON, R. (1923).

The possible significance of hexose-phosphoric esters in ossification.

Biochem. J. 17, 286-293.

ROHLIN, M. (1976).

Whole-body autoradiography of $^{99}\text{Tc}^{\text{m}}$ -labelled ethylene-1-hydroxy-1,1-diphosphonate (EHDP) in young rats.

Acta Radiol. 15, 410-416.

ROHLIN, M. and HAMMERSTROM, L. (1976).

Whole-body autoradiography of $^{99}\text{Tc}^{\text{m}}$ -labelled pyrophosphate and related compounds in young rats.

Acta Radiol. 15, 71-80.

ROSE, M.B. (1975).

Renal stone formation. The inhibitory effect of urine on calcium oxalate precipitation.

Invest. Urol. 12, 428-433.

ROWE, D.J. and HAUSMANN, E. (1976).

The alteration of osteoclast morphology by disphosphonate in bone organ culture.

Cal. Tiss. Res. 20, 53-60.

RUSSELL, R.G.G. and HODGKINSON, A. (1966).

The urinary excretion of inorganic pyrophosphate by normal subjects and patients with renal calculus.

Clin. Sci. 31, 51-62.

RUSSELL, R.G.G.,

—— MUHLBAUER, R.C., BISAZ, S., WILLIAMS, D.A. and FLEISCH, H. (1970).

The influence of pyrophosphate, condensed phosphates, phosphonates and other phosphate compounds on the dissolution of hydroxyapatite in vitro and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomised rats. Cal. Tiss. Res. 6, 183-196.

—— DOUGLAS, D.L., DUCKWORTH, T., DANIS, J.A., PRESTON, C.J., BAUMANN, T.M. and KLEISCH, H. (1981).

Treatment of metabolic bone and stone diseases with diphosphonate.

In: "Urinary Calculus", J.G. Brockis and B. Finlayson (eds.) pp 477-489

PSG Publishing Co., Massachusetts.

SADLER, R. and SHAW, M. (1978).

A caution against the use of polyvinylpyrrolidone in the extraction of plant glutamic synthetase. Can. J. Bot. 56, 1382-1385.

SALLIS, J.D. and LUMLEY, M.F. (1979).

On the possible role of glycosaminoglycans as natural inhibitors of calcium oxalate stones.

Invest. Urol. 16, 296-299.

SANTI, W., HUEN, J.M. and FREI, R.W. (1975).

High-speed ion-pair partition chromatography in pharmaceutical analysis.

J. Chr. 115, 423-436.

SARGEANT, L.E. and STINSON, R.A. (1979).

Inhibition of human alkaline phosphatases by vanadate.

Biochem. J. 81, 247-250.

SCHILLER, S., SLOVER, G.A. and DORFMAN, P.R. (1961).

A method for the separation of acid mucopolysaccharides: Its application to the isolation of heparin from the skin of rats.

J. Biol. Chem. 236, 983-987.

SCHLESINGER, D.H. and HAY, D.I. (1977).

Complete covalent structure of statherin a tyrosine-rich acidic peptide which inhibits calcium phosphate precipitation from human parotid saliva.

J. Biol. Chem. 252, 1689-1695.

SCHRAER, H. and GAY, C.V. (1977).

Matrix vesicles in newly synthesizing bone observed after ultracryotomy and ultramicro-incineration.

Cal. Tiss. Res. 23, 185-188.

SCOTT, R.P.W. (1978).

Highlights from contemporary analytical liquid chromatography.

Analyst 103, 37-55.

SHAPIRO, I.M. and LEE, N.M. (1978).

The effect of oxygen, phosphoenol pyruvate and pH on the release of calcium from chondrocyte mitochondria.

Metab. Bone Dis. Rel. Res. 1, 173-177.

SHAPIRO, I.M., BURKE, A. and LEE, N.M. (1976).

Heterogeneity of chondrocyte mitochondria. A study

of the Ca^{++} concentration and density banding characteristics of normal and rachitic cartilage.

Biochem. Biophys. acta 451, 583-591.

SINGLA, S.K., GOYAL, S.C., SINGH, K.P. and JETHI, R.K. (1978).

Anticalcifying properties of urine from normal persons and kidney stone patients.

Indian J. Med. Res. 68, 695-699.

SJODIN, B., KORWILLEM, A. and KARLSSON, J. (1975).

Isotachophoresis: A new technique for determination of tissue metabolite concentration.

Protides and Biol. Fluids. 22, 733-736.

SLOTIN, L.A. (1977).

Current methods of phosphorylation of biological molecules.

Synth. 737-752.

SMITH, L.H., MEYER, J.L. and McCALL, J.T. (1973).

Chemical nature of crystal inhibitors isolated from human urine.

In: "Urinary Calculi", Int. Symp. Renal Stone Res., Madrid, 1972.

pp 318-327, Karger, Basel.

SOWADSKI, I.J., CORNICK, G. and KRETSINGER, R.H. (1978):

Terbium replacement of calcium in parvalbumin.

J. Mol. Biol. 124, 123-132.

STAGNI, N., FURLAN, G., VITTUR, F., ZANETTI, M. and de BERNARD, B. (1979).

Enzymatic properties of the Ca^{2+} -binding glycoprotein isolated from preosseous cartilage.

Cal. Tiss. Int. 29, 27-32.

STRATES, B.S., FIRSCHEIN, H.E. and URIST, M.R. (1971).

Alkaline phosphatase and failure of calcification under the influence of a diphosphonate.

Biochem. Biophys. acta 244, 121-124.

SUKI, W.N. (1979).

Calcium transport in the nephron.

Am. J. Physiol. 237, F1-F6.

SUTOR, D.J. (1969).

Growth studies of calcium oxalate in the presence of various ions and compounds.

Brit. J. Urol. 41, 171-178.

SUTOR, D.J. and PERCIVAL, J.M. (1978).

An assay method for the measurement of urinary inhibitors of calcium phosphate formation.

Clin. Chim. acta 89, 267-272.

SUTOR, D.J.,

—— PERCIVAL, J.M. and DOONAN, S. (1978).

Isolation and identification of some urinary inhibitors of calcium phosphate formation.

Clin. Chim. acta 89, 273-278.

—— PERCIVAL, J.M. and DOONAN, S. (1979a).

Urinary inhibitors of the formation of calcium oxalate.

Brit. J. Urol. 51, 253-255.

—— PERCIVAL, J.M. and PIPER, K.A. (1979b).

Urinary inhibitors of calcium phosphate formation: the inhibitory activity of normal and artificial urine.

Brit. J. Urol. 51, 1-5.

TAVES, D.R. and REEDY, R.C. (1969).

A structural basis for the transphosphorylation of nucleotides with hydroxyapatite.

Cal. Tiss. Res. 3, 284-292.

TAYLOR, T.G. (1953).

A modified procedure for the microdetermination of citric acid.

Biochem. J. 54, 48-49.

TEITELBAUM, S.L. and KAHN, A.J. (1980).

Mononuclear phagocytes, osteoclasts and bone resorption.

Min. Elect. Metab. 3, 2-9.

TENER, G.M. (1961).

2-cyanoethyl phosphate and its use in the synthesis of phosphate esters.

J.A.C.S. 83, 159-168.

TERMINE, J.D. and CONN, K.M. (1976).

Inhibition of apatite formation by phosphorylated metabolites and macromolecules.

Cal. Tiss. Res. 22, 149-157.

TERMINE, J.D. and EANES, E.D. (1972).

Comparative chemistry of amorphous and apatitic calcium phosphate preparations.

Cal. Tiss. Res. 10, 171-197.

TERMINE, J.D. and POSNER, A.S. (1967).

Amorphous/crystalline inter-relationships in bone mineral.

Cal. Tiss. Res. 1, 8-23.

TERMINE, J.D. and POSNER, A.S. (1970).

Calcium phosphate formation in vitro. I. Factors affecting initial phase separation.

Arch. Biochem. Biophys. 140, 307-317.

TERMINE, J.D.,

——— PULLMAN, I. and POSNER, A.S. (1967).

Electron-spin resonance study of irradiated bone and its constituents.

Arch. Biochem. Biophys. 122, 318-330.

——— PECKAUSKAS, R.A. and POSNER, A.S. (1970).

Calcium phosphate formation in vitro. II. Effects of environment on amorphous-crystalline transformation.

Arch. Biochem. Biophys. 140, 318-325.

——— EANES, E.D. and CONN, K.M. (1980).

Phosphoprotein modulation of apatite crystallization.

Cal. Tiss. Int. 31, 247-251.

TERNYNCK, T. and AVRAMEAS, S. (1976).

A new method using p-benzoquinone for coupling antigens and antibodies to marker substances.

Ann. Immunol. (Inst. Pasteur) 127C, 197-208.

TEW, W.P. (1977).

Use of the coulombic interaction of the lanthanide series to identify two classes of Ca^{++} binding sites in mitochondria.

Biochem. Biophys. Res. Comm. 78, 624-630.

TEW, W.P. and MAHLE, C. (1977).

Isolation of an inhibitor of calcification from rat liver mitochondria.

Fed. Proc. 36, 859.

TEW, W.P. and MALIS, C.D. (1981).

Calcium electrode measurements of calcium oxalate mineralization: the effects of urine and other inhibitors.

Proceedings of the Fourth Int. Symp, on Urolithiasis, July 1980.

In: "Urolithiasis" pp 321-333

Plenum Press, U.S.A.

TEW, W.P.,

——— MAHLE, C. and LEHNINGER, A.L. (1978).

Identification of phosphocitrate, a naturally occurring inhibitor of biological calcification.

Biophys. J. 21, 60a.

——— MAHLE, C., BENAVIDES, J., HOWARD, J.E. and LEHNINGER, A.L. (1980).

Synthesis and characterization of phosphocitric acid, a potent inhibitor of hydroxyapatite crystal growth.

Biochem. 19, 1983-1988.

——— MALIS, C.D., LEHNINGER, A.L., WALKER, G.W. and HOWARD, J.E. (1981).

Inhibition of PTH-induced nephrocalcin by phosphocitrate.

Proceedings of the Fourth Int. Symp. on Urolithiasis, July, 1980.

p 559-568

Plenum Press, U.S.A.

THOMAS, W.C. (1971).

Effectiveness and mode of action of orthophosphates in patients with calcareous renal calculi.

Trans. Amer. Clin. Climat. Ass. 83, 113-124.

THOMAS, M.L. and RAMP, W.K. (1978).

Effects of parathyroid hormone on alkaline phosphatase activity and mineralization of cultured chick embryo tibia.

Cal. Tiss. Res. 27, 137-142.

THOMMASET, M., REDEL, J., MARCHE, P. and CUISINIER-
GLEIZES, P. (1978).

Duodenal calcium-binding protein (CaBP) and bone calcium mobilisation in response to 24R, 25 and 24S, 25-dihydroxycholecalciferol in intact and nephrectomized rats.

J. Steroid Biochem. 9, 159-162.

TOCHON-DANGUY, H.J., GEOFFROY, M. and BAUD, C.A. (1980).

Electron-spin resonance study of the effects of carbonate substitution in synthetic apatites and apatites from human teeth.

Arch. oral Biol. 25, 357-361.

TOMLINSON, E., JEFFERIES, T.M. and RILEY, C.M. (1978).

Ion-pair high-performance liquid chromatography.

J. Chr. 159, 315-358.

TURKELSON, V.J. and RICHARDS, M. (1978).

Separation of the citric acid cycle acids by liquid chromatography.

Anal. Chem. 50, 1420-1423.

VAN NEUTEN, J.M. and WELLENS, D. (1979).

Mechanisms of vasodilation and antivasoconstriction.

Angiology 30, 440.

VIGNERY, A. and BARON, R. (1978).

Effects of parathyroid hormone on the osteoclastic pool, bone resorption and formation in rat aveolar bone.

Cal. Tiss. Res. 26, 23-28.

VOGEL, J.J., BOYAN-SALYERS, B. and CAMPBELL, M.M. (1978).

Protein-phospholipid interactions in biologic calcification.

Metab. Bone Dis. Rel. Res. 1, 149-153.

WADDELL, W.J. (1972).

A molecular mechanism for biological calcification.

Biochem. Biophys. Res. Comm. 49, 127-132.

WADKINS, C.L. and LUBEN, R.A. (1978).

Effects of fluoride on in vitro calcification of tendon matrix.

Cal. Tiss. Res. 26, 51-59.

WALSH, C.T. (1970).

Citryl phosphate and the mechanism of the citrate cleavage reaction.

Diss. Abstr. Int. 31, 7162B.

WALSH, C.J. and SPECTOR, L.B. (1968).

Citryl-1-phosphate and the citrate cleavage reaction.

J. Biol. Chem. 243, 446-451.

WALSH, C.J. and SPECTOR, L.B. (1969).

Citryl phosphate and the mode of action of the citrate cleavage enzyme.

J. Biol. Chem. 244, 4366-4374.

WELSHMAN, S.G. and McGEOWN, M.G. (1972).

A quantitative investigation of the effects on the growth of calcium oxalate crystals on potential inhibitors.

Brit. J. Urol. 44, 677-680.

WERINGER, E.J., OLDHAM, S.B. and BETHUNE, J.E. (1978).

A proposed cellular mechanism for calcium transport in the intestinal epithelial cell.

Cal. Tiss. Res. 26, 71-79.

WHEELER, E.J. and LEWIS, D. (1977).

An X-ray study of the paracrystalline nature of bone apatite.

Cal. Tiss. Res. 24, 243-248.

WIEDNER, G. and WILHELM, G. (1977).

Calcification of a native collagen membrane.

Z. Naturforsch. 32c, 997-1002.

WILKES, J.S., HAPKE, B. and LETSINGER, R.L. (1973).

A 5'-amino analog of adenosine diphosphate.

Biochem. Biophys. Res. Comm. 53, 917-922.

WILLIAMS, G. and SALLIS, J.D. (1979).

Structure-activity relationship of inhibitors of hydroxyapatite formation.

Biochem. J. 184, 181-184.

WILLIAMS, G. and SALLIS, J.D. (1980).

The synthesis of unlabelled and ^{32}P -labelled phosphocitrate and analytical systems for its identification.

Anal. Biochem. 102, 365-373.

WILLIAMS, G. and SALLIS, J.D. (1981a).

The sources of phosphocitrate and analytical systems for its identification.

Proceedings of the Fourth Int. Symp. on Urolithiasis, July, 1980.

In: "Urolithiasis", p 321-333

Plenum Press, U.S.A.

WILLIAMS, G. and SALLIS, J.D. (1981b).

Structural factors influencing the ability of compounds to inhibit hydroxyapatite formation.

Cal. Tiss. Int. (in press).

WILLIAMS, G. and SALLIS, J.D. (1981c).

Identification of phosphocitrate in soft tissue mitochondria.

Proc. Aust. Biochem. Soc. 14, 45.

WILLIAMS, G. and SALLIS, J.D. (1981d).

The fate of intravenously administered phosphocitrate, a potent inhibitor of calcification.

Clin. Exp. Pharm. Physiol. (in press).

WILLIAMS, G., HOLDSWORTH, E.S. and SALLIS, J.D. (1980).

Phosphocitrate: a new factor in the control of biological calcification.

Clin. Exp. Pharm. Physiol. 7, 42.

WONG, R.S.C. and BENNICK, A. (1980).

The primary structure of a salivary calcium-binding proline-rich phosphoprotein (Protein C), a possible precursor of a related salivary protein A.

J. Biol. Chem. 255, 5943-5948.

WUTHIER, R.E. and EANES, E.D. (1975).

Effect of phospholipids on the transformation of amorphous calcium phosphate to hydroxyapatite in vitro.

Cal. Tiss. Res. 19, 197-210.

WUTHIER, R.E. and GORE, S.T. (1977).

Partition of inorganic ions and phospholipids in isolated cell, membrane and matrix vesicle fractions: Evidence for Ca-P_i -acidic phospholipid complexes.

Cal. Tiss. Res. 24, 163-171.

WUTHIER, R.E., MAJESKA, R.J. and COLLINS, G.M. (1977).

Biosynthesis of matrix vesicles in epiphyseal cartilage. I. In vivo incorporation of ^{32}P -orthophosphate into phospholipids of chondrocyte, membrane and matrix vesicle fractions.

Cal. Tiss. Res. 23, 135-139.

YAMADA, M. (1976).

Ultrastructural and cytochemical studies on the calcification of the tendon-bone joint.

Achiv. hist. jap. 39, 347-378.

YAMAGATA, T., SAITO, H., HABUCHI, O. and SUZUKI, S. (1968).

Purification and properties of bacterial chondroitinases and chondrosulfatases.

J. Biol. Chem. 243, 1523.

YAMAZAKI, R.K., MICKEY, D.L. and STORY, M. (1979).

The calibration and use of a calcium ion-specific electrode for kinetic studies of mitochondrial calcium transport.

Anal. Biochem. 93, 430-441.

YOUNT, R.G. (1974).

Adenylylimidodiphosphate and guanylylimidodiphosphate.

Meth. Enz. 138C, 420-427.

YOUNT, R.G., BABCOCK, D., BALLATYNE, W. and OJALA, D.
(1971).

Adenylyl imidodiphosphate, an adenosine triphosphate
analog containing a P-N-P linkage.

Biochem. 10, 2484-2495.

ZHDANOV, R.I. and ZHENODAROVA, S.M. (1975).

Chemical methods of oligonucleotide synthesis.

Synth. 222-245.

ZWEIG, G. and SHERMA, J. (1972).

"Handbook of Chromatography", Vol. II.

CRC Press, Ohio, U.S.A.

These articles have been removed for copyright or proprietary reasons.

Williams, G., Sallis, J.D., 1980, The synthesis of unlabeled and ^{32}P -labeled phosphocitrate and analytical systems for its identification, *Analytical biochemistry*, 102(2), 365-373

Williams, G., Sallis, J.D., 1979, Structure-activity relationship of inhibitors of hydroxyapatite formation, *Biochemical journal*, 184(1), 181-184

THE SOURCES OF PHOSPHOCITRATE AND ITS ROLE AS AN INHIBITOR OF CALCIUM PHOSPHATE AND CALCIUM OXALATE CRYSTALLIZATION

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Earlier studies reported by Howard¹ and Lehninger² suggested that a substance containing P_i and citrate, and tentatively assigned the structure of phosphocitrate, existed in nature and was a potent inhibitor of hydroxyapatite (HAP) formation. These observations led us to attempt to isolate and characterize phosphocitrate from biological sources with a view to defining a physiological role for this compound in calcification systems.

As an initial step toward achieving this goal, we have recently synthesized and characterized labelled and unlabelled phosphocitrate³. During the developmental period, systems for monitoring reactions and identifying phosphocitrate in biological studies were established. Whilst NMR data (1H -NMR, ^{13}C -NMR, ^{31}P -NMR) together with mass spectrometry provide information on the structural nature of a compound, with biological samples it is not always possible to obtain sufficient amounts of pure material for analysis by these methods. Instead, we have found that a range of techniques is more valuable for characterizing phosphocitrate than any single method, and a summary of systems that we are now using is presented in Table 1.

Ion exchange chromatography at neutral pH enables preliminary separation of biological samples with minimal hydrolysis and eliminates phosphocitrate artefact formation. Thin layer chromatography, high voltage electrophoresis, enzymatic and chemical analyses provide versatility for identification. Currently, we are investigating the application of analytical isotachopheresis for identification and quantitation of phosphocitrate as analysis is rapid, and separation of phosphocitrate from interfering ions or compounds appears complete.

Synthetic phosphocitrate has been tested *in vitro* for its ability to inhibit HAP and calcium oxalate crystal formation. By simultaneous measurement of alkali addition, calcium ion concentration and laser beam light scattering, information on the relative ability of compounds to inhibit the transformation of amorphous calcium phosphate (ACP) into HAP can be assessed. Comparative studies have shown that phosphocitrate is the most potent of many inhibitors tested including ethane-1-hydroxy-1, 1-diphosphonate (EHDP), ATP and pyrophosphate^{4,5}. Data derived from this system have proved useful in defining the structural requirements of a compound for inhibition.

More recently, our studies with the system have been extended and light scattering data (Figure 1) used to demonstrate that inhibitors may be classified into two groups, Type I and Type II. Type I are all crystal growth inhibitors and, at lower concentrations result in the formation of highly dispersed HAP suspensions, as indicated by an increase in the turbidity change. At higher concentrations the extent of conversion into HAP is inhibited. In contrast, Type II compounds do not inhibit crystal growth nor alter the turbidity measurements or extent of conversion. Electron microscopic observations have substantiated these conclusions. As examples, Type I (in order of potency) includes phosphocitrate, EHDP, tripolyphosphate, imidodiphosphate and pyrophosphate whereas Type II includes ATP, ADP, phosphonoformate, 2-phosphoglyceric acid, 3-phosphoglyceric acid and citrate. Although such physicochemical studies are well defined *in vitro*, it is of questionable validity to extrapolate the results to the physiological situation. For example, inhibitors can, under certain conditions, interact synergistically to inhibit HAP formation.

If phosphocitrate is to play an influential role in urolithiasis, it should inhibit calcium oxalate crystallization. We have studied the influence of phosphocitrate on calcium oxalate crystal growth, using both calcium ion monitoring systems and growth of calcium oxalate crystals on glass fibers. With the latter system, 300 μM phosphocitrate inhibits growth of the crystals to an extent of $66 \pm 3\%$ (relative to buffer); 1 mM pyrophosphate $49 \pm 4\%$, and 100 μM chondroitin sulphate C $55 \pm 7\%$. As with other inhibitors, crystal morphology changes noticeably in their presence.

With phosphocitrate established as a potent inhibitor of calcification and with the availability of systems for the isolation and detection of the compound, we next turned our attention to possible tissue sources. Rabbit liver and kidney homogenates were acid extracted and, after neutralization, subjected to ion exchange chromatography. Following batch elution of P_i and citrate, a gradient was applied, and fractions revealing the presence of citrate after hydrolysis were found to elute with the same elution volume as

Table 1. Techniques for Identification of Phosphocitrate

<u>System</u>	<u>Details</u>	<u>Comments</u>
1. ion-exchange chromatography	AG1-X8, HCO_3^- ; pH 7.4 (ref.3)	batch or gradient with definitive separation of P_i , citrate & PC.
2. thin layer chromatography	cellulose; isobutyric/5% NH_3 /methanol (66:40:100) (ref.3)	R_f values; PC (0.20), P_i (0.44), citrate (0.42).
3. high voltage electrophoresis	paper; 0.05M triethanolamine- HCO_3^- , pH 7.5; 2,000V/2 hr.	distance from origin in cm. PC (23), P_i (32), citrate (25).
4. enzymatic	coupled assay with phosphatase, citrate lyase and malic dehydrogenase; U.V. or fluorimetric (ref.3)	PC sensitivity; 0.5 - 10 ug.
5. chemical analysis	pentabromoacetone derivative (ref.3) benzidine chromaphore (ref.4)	PC sensitivity; 0.5 - 5 ug.
6. isotachopheresis	leading electrolyte; 0.005M HCl-aniline, 0.2% HPMC. trailing electrolyte: 0.005M glutamic, 0.02% HPMC, conductimetric.	step height in cm. Cl (0), PC (30), citrate (45), P_i (60), glutamate (95). PC sensitivity. pmoles, nmoles.

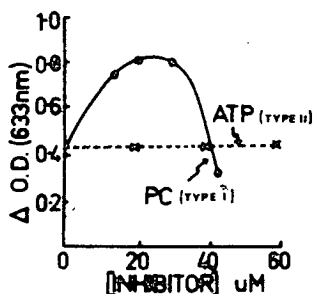


Figure 1. Effect of inhibitor concentration on the magnitude of the turbidity change associated with the transformation of amorphous calcium phosphate to hydroxyapatite.

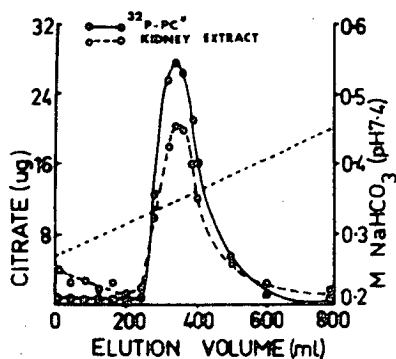


Figure 2. Separation of phosphocitrate from a kidney extract by ion exchange chromatography. Column 10 x 1.5 cm; AG1-X8, HCO_3^- . Batch elution with 0.27M NaHCO_3 pH 7.4, then gradient elution as shown above (----). *Radioactivity profile has been superimposed.

synthetic phosphocitrate (Figure 2). We have no evidence that citrate and P_i together lead to phosphocitrate production under these conditions. Enzymatic analysis provided additional evidence that the compound isolated was phosphocitrate for only when the compound was preincubated with a phosphatase was it possible to obtain a reaction with citrate lyase. Although citryl-1-phosphate (i.e. phosphate esterified to a carboxyl group) might meet the enzyme requirements, stability data eliminate the possibility, as this compound, relative to phosphocitrate, is short-lived³. In additional experiments, isolated phosphocitrate subjected to thin layer chromatography showed only a single band which co-chromatographed with the reference standard and, when hydrolyzed, gave citrate and P_i .

This demonstration that phosphocitrate does exist in tissues, led us to examine the suggestion² that the mitochondria might be an important source of this compound. Mitochondria from rabbit liver, kidney and intestine were found to contain phosphocitrate and, on preliminary estimates, have 0.5, 0.8 and 4.2 nmol phosphocitrate/mg mitochondrial protein respectively. More recent experiments, however, have led us to doubt the validity of the quantitative data as it is now clear that enzymatic hydrolysis is very active in tissues. If a similar situation occurs during isolation of the mitochondria, it might lead to an under-estimation of the true concentration. Further, the status of the mitochondria or indeed the whole animal with regard to calcium and phosphate levels could influence the value obtained. Phosphocitrate has been detected but not yet quantitated in the skeleton and, at present, we have no evidence for its existence in urine. Techniques are being investigated which will allow quantitation of phosphocitrate in bone, where presently, hydroxyapatite avidly binds the compound. In urine, metabolites and salts have so far made investigation of this possible source more difficult.

In order to help define sites of action of phosphocitrate, [^{32}P]-phosphocitrate was injected intravenously into rats and the time course of the accumulation of radioactivity in selected tissues was followed. The results are shown in Figure 3a. After 30 minutes, the majority of the radioactivity was associated with the skeleton (40%) and kidney (15%) whereas liver had only 5% of the initial radioactivity. These results alone, however, do not allow interpretation of the distribution of phosphocitrate in tissue or of its specific binding. To have meaning, the radioactivity must be associated with phosphocitrate and not its hydrolyzed product, P_i . Two approaches have been made to resolve this problem.

In the first instance, [^{32}P]- P_i was injected into rats to determine rates of tissue labelling over the same time period, and to assess the affinity of the label for the skeleton (Figure 3b). Results indicate that only 20% radioactivity from [^{32}P]- P_i was taken up by the skeleton in comparison to 40% when [^{32}P]-phosphocitrate was injected. Kidney shows a similar pattern whereas for liver, the

relationship was reversed with minimal radioactivity accumulating after $[^{32}\text{P}]$ -phosphocitrate was given.

In the second approach, acid extracts of soft tissue were prepared, the radioactivity associated with the organic and inorganic phosphate determined⁷, and the labelled metabolites further resolved and quantitated using high voltage electrophoresis. The organic phosphate to P_i ratio shows that after 30 minutes, 80% of the radioactivity in blood was in the organic phosphate fraction. Electrophoretic patterns (Figures 4 and 5) indicate that this radioactivity was associated with phosphocitrate. By contrast, the kidney has 40% represented as organic phosphates after 5 minutes, and electrophoresis indicates that only 15% of the radioactivity was due to

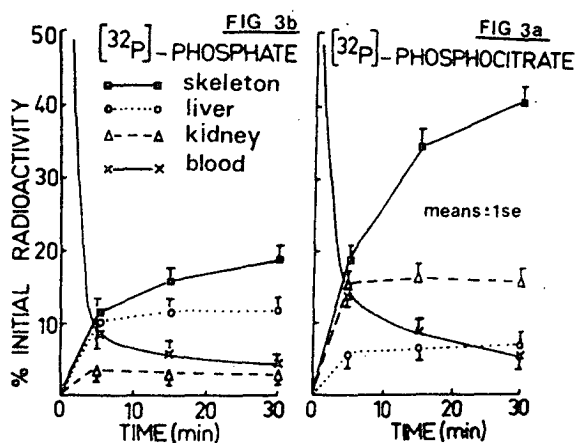
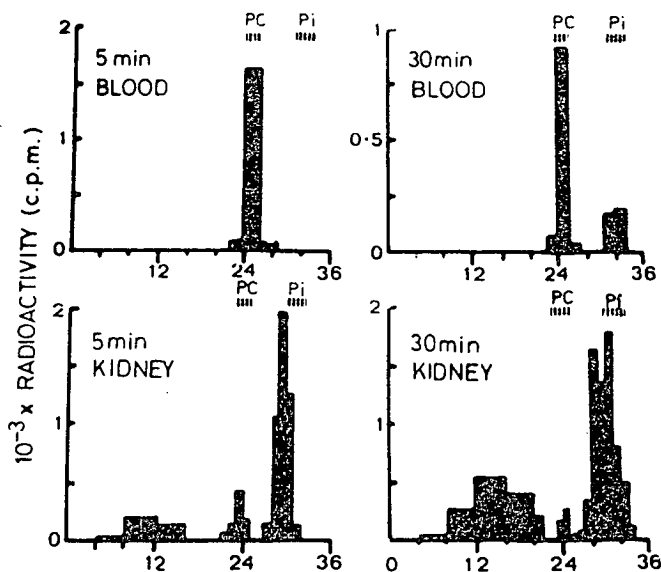
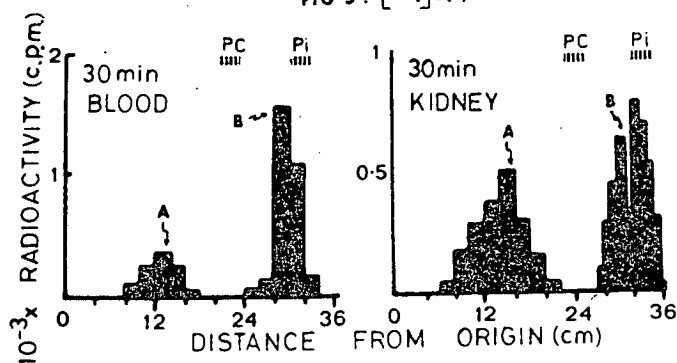


Figure 3. Rate of disappearance of radioactivity in blood and its appearance in tissues following administration of $[^{32}\text{P}]$ - P_i or $[^{32}\text{P}]$ -phosphocitrate in rats. Female rats (170 g) were injected intravenously with 1 mg of the sodium salts of the labelled compounds. At designated times, samples of blood, bone and soft tissues were taken, oxidized and counted. Values shown are means \pm 1 S.E. Total weights were obtained for liver and kidney; skeletal mass was calculated⁸ and the total blood volume assessed at 64 ml/kg.

FIG 4: $[^{32}\text{P}]$ -PHOSPHOCITRATEFIG 5: $[^{32}\text{P}]$ - P_i 

Figures 4 and 5. Radioactive patterns for electrophoresis. Rats were injected intravenously with 1 mg (0.5 μCi) of either $[^{32}\text{P}]$ -phosphocitrate (specific activity = 200 $\mu\text{Ci}/\text{mmol}$ or $[^{32}\text{P}]$ - P_i (specific activity = 70 $\mu\text{Ci}/\text{mmol}$) and at time intervals, relevant samples were collected, frozen and then extracted with trichloroacetic acid. Following neutralization, aliquots were taken for high voltage electrophoresis as described in the text. The dried paper was marked in a grid pattern, then cut, and after acid extraction, radioactivity (■) associated with the separated compounds was determined. Phosphocitrate and P_i were identified from reference standards (|||||) treated in an identical manner.

phosphocitrate. Even less (about 5% of phosphocitrate) was found in kidney after 30 minutes, as shown in Figure 4. Because of the contrast in lability of phosphocitrate between blood and kidney, it is possible that special conditions prevail in the kidney for phosphatase action, or that there exists a specific phosphocitrate cleavage enzyme which rapidly inactivates the molecule. It should be noted that no other labelled compounds were detected of the same electrophoretic mobility as authentic phosphocitrate in tissue extracts from rats injected with [^{32}P]- P_i (Figure 5).

In order to determine whether (a) phosphocitrate was being metabolized to organic phosphate esters or (b) phosphocitrate was being hydrolyzed into P_i which was then incorporated into organic phosphate esters, samples from the [^{32}P]- P_i experiment were subjected to an identical electrophoretic analysis (Figure 5). Liver (not shown here) rapidly metabolized [^{32}P]- P_i into several organic phosphates whereas blood and kidney formed two major organic compounds, shown as A and B in Figure 5. In experiments using [^{32}P]-phosphocitrate in place of [^{32}P]- P_i , both components A and B were discernable in kidney after 30 minutes (Figure 4).

The data, we believe, support the contention that phosphocitrate is hydrolyzed to P_i and citrate before any further metabolism occurs. Due to the avid binding of phosphocitrate in bone, as yet we have been unable to identify the radioactive compound(s) in bone. However, based on the electrophoretic analysis of blood and the relatively low uptake (20%) of [^{32}P]- P_i itself, we believe that the radioactivity found in bone is a true indication of phosphocitrate uptake. Once adsorbed onto HAP, the further fate of phosphocitrate is difficult to determine experimentally.

Exogenous phosphocitrate, then, is localized in at least two tissues - bone and kidney, but its physiological potency at least two sites must be dependent upon its stability in the microenvironment. Our *in vivo* data suggest that at the kidney site, phosphocitrate is rapidly inactivated. If however, endogenous phosphocitrate is strictly compartmentalized within the mitochondria, the lability data from whole tissue may be of limited meaning. Clearly it is important to gain further information regarding the intracellular distribution of phosphocitrate and to elucidate its biological site(s) of synthesis and degradation. The availability of a stable analogue of phosphocitrate capable of resisting enzyme attack would also be valuable. We are currently investigating the synthesis of two such compounds since their biological inertness should permit advantage to be taken, at the kidney site, of the physicochemical properties associated with the parent molecule, phosphocitrate. Because of the possible lability of phosphocitrate in bone, this compound may be more desirable in the treatment of skeletal disorders than an inert analogue.

Whilst, from the data presented here, a physiological role for phosphocitrate in the maintenance of mitochondrial calcium phosphate deposits in the amorphous state is easily envisaged, a much broader role for this molecule in the control of stone formation and other calcifying systems is foreseen but has yet to be elucidated.

REFERENCES

1. J. E. Howard, Johns Hopkins Med. J. 139:239 (1976).
2. A. L. Lehninger, Horiz. Biochem. Biophys. 4:1 (1977).
3. G. Williams and J. D. Sallis, Anal. Biochem. 102:365 (1980).
4. G. B. Jones, Anal. Biochem. 21:286 (1967).
5. G. Williams, E. S. Holdsworth and J. D. Sallis, Clin. Exp. Pharm. Physiol. (in press).
6. G. Williams and J. D. Sallis, Biochem. J. 184:181 (1979).
7. S. T. Ohnishi, Anal. Biochem. 86:201 (1978).
8. H..H. Donaldson, Am. J. Anat. 26:237 (1920).

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